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Date: 2-8-02 Express Mail Label No. EV 05202797548

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Attorney's Docket No.: 3298.1001-000

MULTIPLE DETERMINANTS FOR METABOLIC PHENOTYPES  
RELATED APPLICATIONS

5 This Application claims the benefit of pending  
provisional application U.S. 60/267,472, filed February 9,  
2001, the entire teachings of which are incorporated  
herein by reference.

BACKGROUND OF THE INVENTION

10 The invention relates to multi-determinant metabolic  
phenotyping. More specifically, the present invention  
relates to the characterization of metabolic phenotypes  
based on phenotypic determinants and applications and uses  
thereof. Phenotypic determinants of the present invention  
include but are not limited to determinants for drug  
15 metabolizing enzymes including the following enzymes,  
CYP1A2, N-acetyltransferase-1 (NAT-1),  
N-acetyltransferase-2 (NAT-2), CYP2D6, CYP2A6, CYP2E1,  
CYP3A4, CYP2C9, CYP2C19, UGTs, SUTs, and GSTs. Methods of  
selecting individual treatment regimes, as well as  
20 selecting candidates for clinical trials are also  
provided.

For the majority of drugs (or xenobiotics)  
administered to humans, their fate is to be metabolized in

the liver, into a form less toxic and lipophilic with their subsequent excretion in the urine. Their metabolism involves two systems (Phase I and Phase II) which act consecutively: Phase I enzymes include the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions as well as carboxylesterase, amindases, epoxide hydrolase, quinine reductase, alcohol and aldehyde dehydrogenase, xanthine oxidase and flavin-containing monooxygenase. These enzymes are localized in the microsomal fraction. Phase II enzymes include the conjugation system which involves at least 5 enzymes including, N-acetyltransferases (NAT), UDP-glucoronyltransferases (UGT), sulfotransferases (SUT), and glutathione-S-transferases (GST). A detailed description of the complex human drug metabolizing systems is provided in Kumar and Surapaneni (Medicinal Res. Rev. (2001) 21(5):397-411) and patent application WO 01/59127 A2.

The metabolism of a drug and its movement through the body (pharmacokinetics) are important in determining its effects, toxicity, and interactions with other drugs. The three processes governing pharmacokinetics are the absorption of the drug, distribution to various tissues, and elimination of drug metabolites. These processes are intimately coupled to drug metabolism, since a variety of metabolic modifications alter most of the physicochemical and pharmacological properties of drugs, including solubility, binding to receptors, and excretion rates. The metabolic pathways which modify drugs also accept a variety of naturally occurring substrates such as steroids, fatty acids, prostaglandins, leukotrienes, and

vitamins. The enzymes in these pathways are therefore important sites of biochemical and pharmacological interaction between natural compounds, drugs, carcinogens, mutagens, and xenobiotics.

5        It has long been appreciated that inherited differences in drug metabolism lead to drastically different levels of drug efficacy and toxicity among individuals. For drugs with narrow therapeutic indices, or drugs which require bioactivation (such as codeine), these  
10       polymorphisms can be critical. Moreover, promising new drugs are frequently eliminated in clinical trials based on toxicities which may only affect a segment of the individuals in a target group. Advances in pharmacogenomics research, of which drug metabolizing  
15       enzymes constitute an important part, are promising to expand the tools and information that can be brought to bear on questions of drug efficacy and toxicity (See Evans, W. E. and R. V. Relling (1999) Science 286: 487-491).

20       Drug metabolic reactions are categorized as Phase I, which functionalize the drug molecule and prepare it for further metabolism, and Phase II, which are conjugative. In general, Phase I reaction products are partially or fully inactive, and Phase II reaction products are the  
25       chief excreted species. However, Phase I reaction products are sometimes more active than the original administered drugs; this metabolic activation principle is exploited by pro-drugs (e. g. L-dopa). Additionally, some nontoxic compounds (e. g. aflatoxin, benzo [a] pyrene) are  
30       metabolized to toxic intermediates through these pathways.

Phase I reactions are usually rate-limiting in drug metabolism. Prior exposure to the compound, or other compounds, can induce the expression of Phase I enzymes however, and thereby increase substrate flux through the metabolic pathways. (See Klassen, C. D., Amdur, M. O. and J. Doull (1996) Casarett and Doull's Toxicology : The Basic Science of Poisons, McGraw-Hill, New York, NY, pp. 113-186; Katzung, B. G. (1995) Basic and Clinical Pharmacology, Appleton and Lange, Norwalk, CT, pp. 48-59; Gibson, G. G. and Skett, P. (1994) Introduction to Drug Metabolism, Blackie Academic and Professional, London.)

Drug metabolizing enzymes (DMEs) have broad substrate specificities. This can be contrasted to the immune system, where a large and diverse population of antibodies is highly specific for their antigens. The ability of DMEs to metabolize a wide variety of molecules creates the potential for drug interactions at the level of metabolism. For example, the induction of a DME by one compound may affect the metabolism of another compound by the enzyme.

DMEs have been classified according to the type of reaction they catalyze and the cofactors involved. The major classes of Phase I enzymes include, but are not limited to, cytochrome P450 and flavin-containing monooxygenase. Other enzyme classes involved in Phase 1-type catalytic cycles and reactions include, but are not limited to, NADPH cytochrome P450 reductase (CPR), the microsomal cytochrome b5/NADH cytochrome b5 reductase system, the ferredoxin/ferredoxin reductase redox pair, aldo/keto reductases, and alcohol dehydrogenases. The



major classes of Phase II enzymes include, but are not limited to, UDP glucuronyltransferase, sulfotransferase, glutathione S-transferase, N-acyltransferase, and N-acetyltransferase.

5 Cytochrome P450 and P450 catalytic cycle-associated enzymes

Members of the cytochrome P450 superfamily of enzymes catalyze the oxidative metabolism of a variety of substrates, including natural compounds such as steroids, fatty acids, prostaglandins, leukotrienes, and vitamins, as well as drugs, carcinogens, mutagens, and xenobiotics. Cytochromes P450, also known as P450 heme-thiolate proteins, usually act as terminal oxidases in multi-component electron transfer chains, called P450-containing monooxygenase systems. Specific reactions catalyzed include hydroxylation, epoxidation, N-oxidation, sulfoxidation, N-, S-, and dealkylations, desulfation, deamination, and reduction of azo, nitro, and N-oxide groups. These reactions are involved in steroidogenesis of glucocorticoids, cortisols, estrogens, and androgens in animals; insecticide resistance in insects; herbicide resistance and flower coloring in plants; and environmental bioremediation by microorganisms. Cytochrome P450 actions on drugs, carcinogens, mutagens, and xenobiotics can result in detoxification or in conversion of the substance to a more toxic product. Cytochromes P450 are abundant in the liver, but also occur in other tissues; the enzymes are located in microsomes. (Graham-Lorence, S. and Peterson, J. A. (1996) FASEB J. 10: 206-214.)

Four hundred cytochromes P450 have been identified in diverse organisms including bacteria, fungi, plants, and animals (Graham-Lorence, supra). The B-class is found in prokaryotes and fungi, while the E-class is found in  
5 bacteria, plants, insects, vertebrates, and mammals. Five subclasses or groups are found within the larger family of E-class cytochromes P450.

All cytochromes P450 use a heme cofactor and share structural attributes. Most cytochromes  
10 P450 are 400 to 530 amino acids in length. The secondary structure of the enzyme is about 70% alpha-helical and about 22% beta-sheet. The region around the heme-binding site in the C-terminal part of the protein is conserved among cytochromes P450. A ten amino acid signature  
15 sequence in this heme-iron ligand region has been identified which includes a conserved cysteine involved in binding the heme iron in the fifth coordination site. In eukaryotic cytochromes P450, a membrane-spanning region is usually found in the first 15-20 amino acids of the  
20 protein, generally consisting of approximately 15 hydrophobic residues followed by a positively charged residue (Graham-Lorence, supra.).

Cytochrome P450 enzymes are involved in cell proliferation and development. The enzymes have roles in  
25 chemical mutagenesis and carcinogenesis by metabolizing chemicals to reactive intermediates that form adducts with DNA (Nebert, D. W. and Gonzalez, F. J. (1987) Ann. Rev. Biochem. 56: 945-993). These adducts can cause nucleotide changes and DNA rearrangements that lead to oncogenesis.  
30 Cytochrome P450 expression in liver and other tissues is

induced by xenobiotics such as polycyclic aromatic hydrocarbons, peroxisomal proliferators, phenobarbital, and the glucocorticoid dexamethasone (Dogra, S. C. et al. (1998) Clin. Exp. Pharmacol. Physiol. 25: 1-9). A

5 cytochrome P450 protein may participate in eye development as mutations in the P450 gene CYP1B1 cause primary congenital glaucoma.

Cytochromes P450 are associated with inflammation and infection. Hepatic cytochrome P450 activities are  
10 profoundly affected by various infections and inflammatory stimuli, some of which are suppressed and some induced (Morgan, E. T. (1997) Drug Metab. Rev. 29: 1129-1188). Effects observed *in vivo* can be mimicked by proinflammatory cytokines and interferons.

15 Autoantibodies to two cytochrome P450 proteins were found in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a polyglandular autoimmune syndrome.

Mutations in cytochromes P450 have been linked to  
20 metabolic disorders, including congenital adrenal hyperplasia, the most common adrenal disorder of infancy and childhood; pseudovitamin deficiency rickets; cerebrotendinous xanthomatosis, a lipid storage disease characterized by progressive neurologic dysfunction,  
25 premature atherosclerosis, and cataracts; and an inherited resistance to the anticoagulant drugs coumarin and warfarin (Isselbacher, K. J. et al. (1994) Harrison's Principles of Internal Medicine, McGraw-Hill, Inc. New York, NY, pp. 1968-1970; Takeyama, K. et al. (1997)  
30 Science 277: 1827-1830; Kitanaka, S. et al. (1998) N.

Engl. J. Med. 338: 653-661). Extremely high levels of expression of the cytochrome P450 protein aromatase were found in a fibrolamellar hepatocellular carcinoma from a boy with severe gynecomastia (feminization) (Agarwal, V. R. (1998) J. Clin. Endocrinol. Metab. 83: 1797-1800).

5 The cytochrome P450 catalytic cycle is completed through reduction of cytochrome P450 by NADPH cytochrome P450 reductase (CPR). Another microsomal electron transport system consisting of cytochrome b5 and NADPH cytochrome b5

10 reductase has been widely viewed as a minor contributor of electrons to the cytochrome P450 catalytic cycle. However, a recent report by Lamb, D. C. et al. (1999 ; FEBS Lett. 462: 283-8) identifies a *Candida albicans* cytochrome P450 (CYP51) which can be efficiently reduced and supported by

15 the microsomal cytochrome b5/NADPH cytochrome b5 reductase system. Therefore, there are likely many cytochromes P450 which are supported by this alternative electron donor system.

Cytochrome b5 reductase is also responsible for the

20 reduction of oxidized hemoglobin (methemoglobin, or ferrihemoglobin, which is unable to carry oxygen) to the active hemoglobin (ferrohemoglobin) in red blood cells. Methemoglobinemia results when there is a high level of oxidant drugs or an abnormal hemoglobin (hemoglobin M)

25 which is not efficiently reduced. Methemoglobinemia can also result from a hereditary deficiency in red cell cytochrome b5 reductase (Reviewed in Mansour, A. and Lurie, A. A. (1993) Am. J. Hematol. 42: 7-12).

Members of the cytochrome P450 family are also closely

30 associated with vitamin D synthesis and catabolism.

Vitamin D exists as two biologically equivalent prohormones, ergocalciferol (vitamin D<sub>2</sub>), produced in plant tissues and cholecalciferol (vitamin D<sub>3</sub>), produced in animal tissues. The latter form, cholecalciferol, is formed upon the exposure of 7-dehydrocholesterol to near ultraviolet light (i.e., 290-310 nm), normally resulting from even minimal periods of skin exposure to sunlight (reviewed in Miller, W. L. and Portale, A. A. (2000) Trends in Endocrinology and Metabolism 11: 315-319).

Both prohormone forms are further metabolized in the liver to 25-hydroxyvitamin D (25(OH)D) by the enzyme 25-hydroxylase. 25(OH)D is the most abundant precursor form of vitamin D which must be further metabolized in the kidney to the active form, 1 $\alpha$ ,25-dihydroxyvitamin D (1 $\alpha$ ,25(OH)<sub>2</sub>D), by the enzyme 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase). Regulation of 1 $\alpha$ ,25(OH)<sub>2</sub>D production is primarily at this final step in the synthetic pathway. The activity of 1 $\alpha$ -hydroxylase depends upon several physiological factors including the circulating level of the enzyme product (1 $\alpha$ ,25(OH)<sub>2</sub>D) and the levels of parathyroid hormone (PTH), calcitonin, insulin, calcium, phosphorus, growth hormone, and prolactin. Furthermore, extrarenal 1 $\alpha$ -hydroxylase activity has been reported, suggesting that tissue-specific, local regulation of 1 $\alpha$ ,25(OH)<sub>2</sub>D production may also be biologically important. The catalysis of 1 $\alpha$ ,25(OH)<sub>2</sub>D to 24,25-dihydroxyvitamin D (24,25(OH)<sub>2</sub>D), involving the enzyme 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase), also occurs in the kidney. 24-hydroxylase

can also use  $25(\text{OH})_2\text{D}$  as a substrate (Shinki, T. *et al.* (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94: 12920-12925 ; Miller, W. L. and Portale, A. A. *supra*; and references within).

- 5 Vitamin D 25-hydroxylase,  $1\alpha$ -hydroxylase, and 24-hydroxylase are all NADPH-dependent, type I (mitochondrial) cytochrome P450 enzymes that show a high degree of homology with other members of the family. Vitamin D 25-hydroxylase also shows a broad substrate
- 10 specificity and may also perform 26-hydroxylation of bile acid intermediates and 25,26, and 27-hydroxylation of cholesterol (Dilworth, F. J. *et al.* (1995) *J. Biol. Chem.* 270: 16766-16774; Miller, W. L. and Portale, A. A. *supra*; and references within).
- 15 The active form of vitamin D ( $1\alpha,25(\text{OH})_2\text{D}$ ) is involved in calcium and phosphate homeostasis and promotes the differentiation of myeloid and skin cells. Vitamin D deficiency resulting from deficiencies in the enzymes involved in vitamin D metabolism (e.g.,  $1\alpha$ -hydroxylase)
- 20 causes hypocalcemia, hypophosphatemia, and vitamin D-dependent (sensitive) rickets, a disease characterized by loss of bone density and distinctive clinical features, including bandy or bow leggedness accompanied by a waddling gait. Deficiencies in vitamin D 25-hydroxylase
- 25 cause cerebrotendinous xanthomatosis, a lipid-storage disease characterized by the deposition of cholesterol and cholestanol in the Achilles' tendons, brain, lungs, and many other tissues. The disease presents with progressive neurologic dysfunction, including postpubescent cerebellar

ataxia, atherosclerosis, and cataracts. Vitamin D 25-hydroxylase deficiency does not result in rickets, suggesting the existence of alternative pathways for the synthesis of 25 (OH) D (Griffin, J. E. and Zerwekh, J. E. (1983) J. Clin. Invest. 72: 1190-1199; Gamblin, G. T. et al. (1985) J. Clin. Invest. 75: 954-960; and W. L. and Portale, A. A. supra).

Ferredoxin and ferredoxin reductase are electron transport accessory proteins which support at least one human cytochrome P450 species, cytochrome P450c27 encoded by the CYP27 gene (Dilworth, F. J. et al. (1996) Biochem. J. 320: 267-71). A *Streptomyces srius* cytochrome P450, CYP104D1, was heterologously expressed in *E. coli* and found to be reduced by the endogenous ferredoxin and ferredoxin reductase enzymes (Taylor, M. et al. (1999) Biochem. Biophys. Res. Commun. 263: 838-42), suggesting that many cytochrome P450 species may be supported by the ferredoxin/ferredoxin reductase pair. Ferredoxin reductase has also been found in a model drug metabolism system to reduce actinomycin D, an antitumor antibiotic, to a reactive free radical species (Flitter, W. D. and Mason, R. P. (1988) Arch. Biochem. Biophys. 267: 632-9).

#### Flavin-containing monooxygenase (FMO)

Flavin-containing monooxygenases (FMO) oxidize the nucleophilic nitrogen, sulfur, and phosphorus heteroatom of an exceptional range of substrates. Like cytochromes P450, FMOs are microsomal and use NADPH and O<sub>2</sub>; there is also a great deal of substrate overlap with cytochromes P450. The tissue distribution of FMOs includes liver, kidney, and lung.

There are five different known isoforms of FMO in mammals (FMO1, FMO2, FMO3, FMO4, and FMOS), which are expressed in a tissue-specific manner. The isoforms differ in their substrate specificities and other properties such as inhibition by various compounds and stereospecificity of reaction. FMOs have a 13 amino acid signature sequence, the components of which span the N-terminal two-thirds of the sequences and include the FAD binding region and the FATGY motif which has been found in many N-hydroxylating enzymes (Stehr, M. et al. (1998) Trends Biochem. Sci. 23: 56-57).

Specific reactions include oxidation of nucleophilic tertiary amines to N-oxides, secondary amines to hydroxylamines and nitrones, primary amines to hydroxylamines and oximes, and sulfur containing compounds and phosphines to S-and P-oxides. Hydrazines, iodides, selenides, and boroncontaining compounds are also substrates. Although FMOs appear similar to cytochromes P450 in their chemistry, they can generally be distinguished from cytochromes P450 *in vitro* based on, for example, the higher heat lability of FMOs and the nonionic detergent sensitivity of cytochromes P450; however, use of these properties in identification is complicated by further variation among FMO isoforms with respect to thermal stability and detergent sensitivity. FMOs play important roles in the metabolism of several drugs and xenobiotics. FMO (FMO3 in liver) is predominantly responsible for metabolizing (S)-nicotine to (S)-nicotine N-1'-oxide, which is excreted in urine. FMO is also involved in S-oxygenation of cimetidine, an H2-



antagonist widely used for the treatment of gastric ulcers. Liver-expressed forms of FMO are not under the same regulatory control as cytochrome P450. In rats, for example, phenobarbital treatment leads to the induction of cytochrome P450, but the repression of FMO1.

Endogenous substrates of FMO include cysteamine, which is oxidized to the disulfide, cystamin, and trimethylamine (TMA), which is metabolized to trimethylamine N-oxide. TMA smells like rotting fish, and mutations in the FMO3 isoform lead to large amounts of the malodorous free amine being excreted in sweat, urine, and breath. These symptoms have led to the designation fish-odor syndrome.

#### Lysyl Oxidase

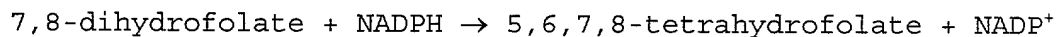
Lysyl oxidase (lysine 6-oxidase, LO) is a copper-dependent amine oxidase involved in the formation of connective tissue matrices by crosslinking collagen and elastin. LO is secreted as a N-glycosylated precursor protein of approximately 50 kDa and cleaved to the mature form of the enzyme by a metalloprotease, although the precursor form is also active. The copper atom in LO is involved in the transport of electron to and from oxygen to facilitate the oxidative deamination of lysine residues in these extracellular matrix proteins. While the coordination of copper is essential to LO activity, insufficient dietary intake of copper does not influence the expression of the apoenzyme. However, the absence of the functional LO is linked to the skeletal and vascular tissue disorders that are associated with dietary copper deficiency. LO is also inhibited by a variety of semicarbazides, hydrazines, and amino nitrites, as well as

heparin. Beta-aminopropionitrile is a commonly used inhibitor. LO activity is increased in response to ozone, cadmium, and elevated levels of hormones released in response to local tissue trauma, such as transforming growth factor-beta, platelet-derived growth factor, angiotensin II, and fibroblast growth factor.

Abnormalities in LO activity has been linked to Menkes syndrome and occipital horn syndrome. Cytosolic forms of the enzyme have been implicated in abnormal cell proliferation (reviewed in Rucker, R. B. et al. (1998) Am. J. Clin. Nutr. 67: 996S-1002S and Smith-Mungo. L. I. and Kagan, H. M. (1998) Matrix Biol. 16: 387-398).

#### 15 Dihydrofolate Reductases

Dihydrofolate reductases (DHFR) are ubiquitous enzymes that catalyze the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, an essential step in the *de novo* synthesis of glycine and purines as well as the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). The basic reaction is as follows:



The enzymes can be inhibited by a number of dihydrofolate analogs, including trimethoprim and methotrexate. Since an abundance of TMP is required for DNA synthesis, rapidly dividing cells require the activity of DHFR. The replication of DNA viruses (i.e.,

herpesvirus) also requires high levels of DHFR activity. As a result, drugs that target DHFR have been used for cancer chemotherapy and to inhibit DNA virus replication. (For similar reasons, thymidylate synthetases are also  
5 target enzymes.) Drugs that inhibit DHFR are preferentially cytotoxic for rapidly dividing cells (or DNA virus-infected cells) but have no specificity, resulting in the indiscriminate destruction of dividing cells. Furthermore, cancer cells may become resistant to  
10 drugs such as methotrexate as a result of acquired transport defects or the duplication of one or more DHFR genes (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 511-5619).

#### 15 Aldo/keto Reductases

Aldo/keto reductases are monomeric NADPH-dependent oxidoreductases with broad substrate specificities (Bohren, K. M. et al. (1989) J. Biol. Chem. 264: 9547-51). These enzymes catalyze the reduction of carbonyl-  
20 containing compounds, including carbonyl-containing sugars and aromatic compounds, to the corresponding alcohols. Therefore, a variety of carbonyl-containing drugs and xenobiotics are likely metabolized by enzymes of this class.

25 One known reaction catalyzed by a family member, aldose reductase, is the reduction of glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. Under normal conditions, the reduction of glucose to sorbitol is a minor pathway. In hyperglycemic  
30 states, however, the accumulation of sorbitol is

implicated in the development of diabetic complications. Members of this enzyme family are also highly expressed in some liver cancers (Cao, D. et al. (1998) J. Biol. Chem. 273: 11429-35).

## 5 Alcohol Dehydrogenases

Alcohol dehydrogenases (ADHs) oxidize simple alcohols to the corresponding aldehydes. ADH is a cytosolic enzyme, prefers the cofactor NAD<sup>+</sup>, and also binds zinc ion. Liver contains the highest levels of ADH, with lower levels in kidney, lung, and the gastric mucosa.

Known ADH isoforms are dimeric proteins composed of 40 kDa subunits. There are five known gene loci which encode these subunits (a, b, g, p, c), and some of the loci have characterized allelic variants (b<sup>2</sup>, b<sup>3</sup>, g<sup>1</sup>, g<sup>2</sup>). The subunits can form homodimers and heterodimers; the subunit composition determines the specific properties of the active enzyme. The holoenzymes have therefore been categorized as Class I (subunit compositions aa, ab, ag, bg, gg), Class II (pp), and Class III (cc). Class I ADH isozymes oxidize ethanol and other small aliphatic alcohols, and are inhibited by pyrazole. Class II isozymes prefer longer chain aliphatic and aromatic alcohols, are unable to oxidize methanol, and are not inhibited by pyrazole. Class III isozymes prefer even longer chain aliphatic alcohols (five carbons and longer) and aromatic alcohols, and are not inhibited by pyrazole.

The short-chain alcohol dehydrogenases include a number of related enzymes with a variety of substrate specificities. Included in this group are the mammalian enzymes D-beta-hydroxybutyrate dehydrogenase, ®-3-

hydroxybutyrate dehydrogenase, 15- hydroxyprostaglandin dehydrogenase, NADPH-dependent carbonyl reductase, corticosteroid 11-beta-dehydrogenase, and estradiol 17-beta-dehydrogenase, as well as the bacterial enzymes

5 acetoacetyl-CoA reductase, glucose 1- dehydrogenase, 3-beta-hydroxysteroid dehydrogenase, 20-beta-hydroxysteroid dehydrogenase, ribitol dehydrogenase, 3-oxoacyl reductase, 2, 3-dihydro-2, 3-dihydroxybenzoate dehydrogenase, sorbitol-6-phosphate 2-dehydrogenase, 7-alpha-

10 hydroxysteroid dehydrogenase, cis-1, 2-dihydroxy-3, 4-cyclohexadiene-1-carboxylate dehydrogenase, cis-toluene dihydrodiol dehydrogenase, cis-benzene glycol dehydrogenase, biphenyl-2, 3-dihydro-2, 3-diol dehydrogenase, N-acylmannosamine 1- dehydrogenase, and 2-

15 deoxy-D-gluconate 3-dehydrogenase (Krozowski, Z. (1994) J. Steroid Biochem. Mol. Biol. 51: 125-130; Krozowski, Z. (1992) Mol. Cell Endocrinol. 84: C25-31; and Marks, A. R. et al. (1992) J. Biol. Chem. 267: 15459-15463).

20 UDP Glucuronyltransferase

Members of the UDP glucuronyltransferase family (UGTs) catalyze the transfer of a glucuronic acid group from the cofactor uridine diphosphate-glucuronic acid (UDP-glucuronic acid) to a substrate. The transfer is generally

25 to a nucleophilic heteroatom (O, N, or S). Substrates include xenobiotics which have been functionalized by Phase I reactions, as well as endogenous compounds such as bilirubin, steroid hormones, and thyroid hormones. Products of glucuronidation are excreted in urine if the

30 molecular weight of the substrate is less than about 250

g/mol, whereas larger glucuronidated substrates are excreted in bile.

UGTs are located in the microsomes of liver, kidney, intestine, skin, brain, spleen, and nasal mucosa, where they are on the same side of the endoplasmic reticulum membrane as cytochrome P450 enzymes and flavin-containing monooxygenases, and therefore are ideally located to access products of Phase I drug metabolism. UGTs have a C-terminal membrane-spanning domain which anchors them in the endoplasmic reticulum membrane and a conserved signature domain of about 50 amino acid residues in their C terminal section.

UGTs involved in drug metabolism are encoded by two gene families, UGT1 and UGT2. Members of the UGT1 family result from alternative splicing of a single gene locus, which has a variable substrate binding domain and constant region involved in cofactor binding and membrane insertion. Members of the UGT2 family are encoded by separate gene loci, and are divided into two families, UGT2A and UGT2B. The 2A subfamily is expressed in olfactory epithelium, and the 2B subfamily is expressed in liver microsomes. Mutations in UGT genes are associated with hyperbilirubinemia; Crigler-Najjar syndrome, characterized by intense hyperbilirubinemia from birth; and a milder form of hyperbilirubinemia termed Gilbert's disease.

#### Sulfotransferase

Sulfate conjugation occurs on many of the same substrates which undergo O-glucuronidation to produce a

highly water-soluble sulfuric acid ester.

Sulfotransferases (ST) catalyze this reaction by transferring  $\text{SO}_3$ -from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the substrate. ST substrates are predominantly phenols and aliphatic alcohols, but also include aromatic amines and aliphatic amines, which are conjugated to produce the corresponding sulfamates. The products of these reactions are excreted mainly in urine.

STs are found in a wide range of tissues, including liver, kidney, intestinal tract, lung, platelets, and brain. The enzymes are generally cytosolic, and multiple forms are often co-expressed. For example, there are more than a dozen forms of ST in rat liver cytosol. These biochemically characterized STs fall into five classes based on their substrate preference: arylsulfotransferase, alcohol sulfotransferase, estrogen sulfotransferase, tyrosine ester sulfotransferase, and bile salt sulfotransferase.

ST enzyme activity varies greatly with sex and age in rats. The combined effects of developmental cues and sex-related hormones are thought to lead to these differences in ST expression profiles, as well as the profiles of other DMEs such as cytochromes P450. Notably, the high expression of STs in cats partially compensates for their low level of UDP glucuronyltransferase activity.

Several forms of ST have been purified from human liver cytosol and cloned. There are two phenol sulfotransferases with different thermal stabilities and substrate preferences. The thermostable enzyme catalyzes the sulfation of phenols such as para-nitrophenol,

minoxidil, and acetaminophen; the thermolabile enzyme prefers monoamine substrates such as dopamine, epinephrine, and levadopa. Other cloned STs include an estrogen sulfotransferase and an N-acetylglucosamine-6-O-sulfotransferase. This last enzyme is illustrative of the other major role of STs in cellular biochemistry, the modification of carbohydrate structures that may be important in cellular differentiation and maturation of proteoglycans. Indeed, an inherited defect in a sulfotransferase has been implicated in macular corneal dystrophy, a disorder characterized by a failure to synthesize mature keratan sulfate proteoglycans (Nakazawa, K. et al. (1984) J. Biol. Chem. 259: 13751-7).

#### Galactosyltransferases

Galactosyltransferases are a subset of glycosyltransferases that transfer galactose (Gal) to the terminal N-acetylglucosamine (GlcNAc) oligosaccharide chains that are part of glycoproteins or glycolipids that are free in solution (Kolbinger, F. et al. (1998) J. Biol. Chem. 273: 433-440; Amado, M. et al. (1999) Biochim. Biophys. Acta 1473: 35-53). Galactosyltransferases have been detected on the cell surface and as soluble extracellular proteins, in addition to being present in the Golgi.  $\beta$ 1,3-galactosyltransferases form Type I carbohydrate chains with Gal ( $\beta$ 1-3) GlcNAc linkages. Known human and mouse  $\beta$ 1,3-galactosyltransferases appear to have a short cytosolic domain, a single transmembrane domain, and a catalytic domain with eight conserved regions.



(Kolbinger, F. *supra* and Hennet, T. *et al.* (1998) *J. Biol. Chem.* 273: 58-65). In mouse UDP-galactose :  $\beta$ -N-acetylglucosamine  $\beta$ 1,3-galactosyltransferase-I region 1 is located at amino acid residues 78-83, region 2 is located at amino acid residues 93-102, region 3 is located at amino acid residues 116-119, region 4 is located at amino acid residues 147-158, region 5 is located at amino acid residues 172-183, region 6 is located at amino acid residues 203-206, region 7 is located at amino acid residues 236-246, and region 8 is located at amino acid residues 264-275. A variant of a sequence found within mouse UDP-galactose: $\beta$ -N-acetylglucosamine  $\beta$ 1,3-galactosyltransferase-I region 8 is also found in bacterial galactosyltransferases, suggesting that this sequence defines a galactosyltransferase sequence motif (Henet, T. *supra*). Recent work suggests that brainiac protein is a  $\beta$ 1,3-galactosyltransferase. (Yuan, Y. *et al.* (1997) *Cell* 88: 9-11; and Henet, T. *supra*).

UDP-Gal:GlcNAc-1, 4-galactosyltransferase (-1, 4-GalT) (Sato, T. *et al.*, (1997) *EMBO J.* 16: 1850-1857) catalyzes the formation of Type II carbohydrate chains with Gal ( $\beta$ 1-4) GlcNAc linkages. As is the case with the  $\beta$ 1,3-galactosyltransferase, a soluble form of the enzyme is formed by cleavage of the membrane-bound form. Amino acids conserved among  $\beta$ 1,4-galactosyltransferases include two cysteines linked through a disulfide-bonded and a putative UDPgalactose-binding site in the catalytic domain (Yadav, S. and Brew, K. (1990) *J. Biol. Chem.* 265: 14163-14169; Yadav, S. P. and Brew, K. (1991) *J. Biol. Chem.* 266: 698-

703; and Shaper, N. L. et al. (1997) J. Biol. Chem. 272: 31389-31399).  $\beta$ 1,4-galactosyltransferases have several specialized roles in addition to synthesizing carbohydrate chains on glycoproteins or glycolipids. In mammals a  $\beta$ 1,4-galactosyltransferase, as part of a heterodimer with cc-lactalbumin, functions in lactating mammary gland lactose production. A  $\beta$ 1,4-galactosyltransferase on the surface of sperm functions as a receptor that specifically recognizes the egg. Cell surface  $\beta$ 1,4-galactosyltransferases also function in cell adhesion, cell/basal lamina interaction, and normal and metastatic cell migration (Shur, B. (1993) Curr. Opin. Cell Biol. 5: 854-863; and Shaper, J. (1995) Adv. Exp. Med. Biol. 376: 95-104).

#### 15 Glutathione S-Transferase

The basic reaction catalyzed by glutathione S-transferases (GST) is the conjugation of an electrophile with reduced glutathione (GSH). GSTs are homodimeric or heterodimeric proteins localized mainly in the cytosol, but some level of activity is present in microsomes as well. The major isozymes share common structural and catalytic properties; in humans they have been classified into four major classes, Alpha, Mu, Pi, and Theta. The two largest classes, Alpha and Mu, are identified by their respective protein isoelectric points; pI ~ 7.5-9.0 (Alpha), and pI ~ 6.6 (Mu). Each GST possesses a common binding site for GSH and a variable hydrophobic binding site. The hydrophobic binding site in each isozyme is specific for particular electrophilic substrates. Specific

amino acid residues within GSTs have been identified as important for these binding sites and for catalytic activity. Residues Q67, T68, D101, E104, and R131 are important for the binding of GSH (Lee, H-C et al. (1995) J. Biol. Chem. 270: 99-109). Residues R13, R20, and R69 are important for the catalytic activity of GST (Stenberg G et al. (1991) Biochem. J. 274: 549-55).

In most cases, GSTs perform the beneficial function of deactivation and detoxification of potentially mutagenic and carcinogenic chemicals. However, in some cases their action is detrimental and results in activation of chemicals with consequent mutagenic and carcinogenic effects. Some forms of rat and human GSTs are reliable preneoplastic markers that aid in the detection of carcinogenesis. Expression of human GSTs in bacterial strains, such as *Salmonella typhimurium* used in the well-known Ames test for mutagenicity, has helped to establish the role of these enzymes in mutagenesis. Dihalomethanes, which produce liver tumors in mice, are believed to be activated by GST. This view is supported by the finding that dihalomethanes are more mutagenic in bacterial cells expressing human GST than in untransfected cells (Thier, R. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 8567-80). The mutagenicity of ethylene dibromide and ethylene dichloride is increased in bacterial cells expressing the human Alpha GST, Al-1, while the mutagenicity of aflatoxin B1 is substantially reduced by enhancing the expression of GST (Simula, T. P. et al. (1993) Carcinogenesis 14: 1371-6). Thus, control of GST activity may be useful in the control of mutagenesis and carcinogenesis.

GST has been implicated in the acquired resistance of many cancers to drug treatment, the phenomenon known as multi-drug resistance (MDR). MDR occurs when a cancer patient is treated with a cytotoxic drug such as cyclophosphamide and subsequently becomes resistant to this drug and to a variety of other cytotoxic agents as well. Increased GST levels are associated with some of these drug resistant cancers, and it is believed that this increase occurs in response to the drug agent which is then deactivated by the GST catalyzed GSH conjugation reaction. The increased GST levels then protect the cancer cells from other cytotoxic agents which bind to GST. Increased levels of A1-1 in tumors has been linked to drug resistance induced by cyclophosphamide treatment (Dirven H. A. et al. (1994) Cancer Res. 54: 6215-20). Thus control of GST activity in cancerous tissues may be useful in treating MDR in cancer patients.

#### Gamma-Glutamyl Transpeptidase

Gamma-glutamyl transpeptidases are ubiquitously expressed enzymes that initiate extracellular glutathione (GSH) breakdown by cleaving gamma-glutamyl amide bonds. The breakdown of GSH provides cells with a regional cysteine pool for biosynthetic pathways. Gamma-glutamyl transpeptidases also contribute to cellular antioxidant defenses and expression is induced by oxidative stress. The cell surface-localized glycoproteins are expressed at high levels in cancer cells. Studies have suggested that the high level of gamma-glutamyl transpeptidases activity present on the surface of cancer cells could be exploited

to activate precursor drugs, resulting in high local concentrations of anticancer therapeutic agents (Hanigan, M. H. (1998) Chem. Biol. Interact. 111-112: 333-42 ; Taniguchi, N. and Ikeda, Y. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72: 239-78 ; Chikhi, N. et al. (1999) Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 122: 367-80).

#### Acyltransferase

N-acyltransferase enzymes catalyze the transfer of an amino acid conjugate to an activated carboxylic group. Endogenous compounds and xenobiotics are activated by acyl-CoA synthetases in the cytosol, microsomes, and mitochondria. The acyl-CoA intermediates are then conjugated with an amino acid (typically glycine, glutamin, or taurine, but also ornithine, arginine, histidine, serine, aspartic acid, and several dipeptides) by N-acyltransferases in the cytosol or mitochondria to form a metabolite with an amide bond. This reaction is complementary to O-glucuronidation, but amino acid conjugation does not produce the reactive and toxic metabolites which often result from glucuronidation.

One well-characterized enzyme of this class is the bile acid-CoA : amino acid N-acyltransferase (BAT) responsible for generating the bile acid conjugates which serve as detergents in the gastrointestinal tract (Falany, C. N. et al. (1994) J. Biol. Chem. 269: 19375-9 ; Johnson, M. R. et al. (1991) J. Biol. Chem. 266: 10227-33). BAT is also useful as a predictive indicator for prognosis of hepatocellular carcinoma patients after partial

hepatectomy (Furutani, M. et al. (1996) Hepatology 24: 1441-5).

### Acetyltransferases

5        Acetyltransferases have been extensively studied for  
their role in histone acetylation. Histone acetylation  
results in the relaxing of the chromatin structure in  
eukaryotic cells, allowing transcription factors to gain  
access to promoter elements of the DNA templates in the  
10       affected region of the genome (or the genome in general).  
In contrast, histone deacetylation results in a reduction  
in transcription by closing the chromatin structure and  
limiting access of transcription factors. To this end, a  
common means of stimulating cell transcription is the use  
15       of chemical agents that inhibit the deacetylation of  
histones (e. g., sodium butyrate), resulting in a global  
(albeit artifactual) increase in gene expression. The  
modulation of gene expression by acetylation also results  
from the acetylation of other proteins, including but not  
20       limited to, p53, GATA-1, MyoD, ACTR, TFIIE, TFIIIF and the  
high mobility group proteins (HMG). In the case of p53,  
acetylation results in increased DNA binding, leading to  
the stimulation of transcription of genes regulated by  
p53. The prototypic histone acetylase (HAT) is Gcn5 from  
25       *Saccharomyces cerevisiae*. Gcn5 is a member of a family of  
acetylases that includes Tetrahymena p55, human Gcn5, and  
human p300/CBP. Histone acetylation is reviewed in  
(Cheung, W. L. et al. (2000) Current Opinion in Cell  
Biology 12 : 326-333 and Berger, S. L (1999) Current  
30       Opinion in Cell Biology 11 : 336-341). Some

acetyltransferase enzymes possess the alpha/beta hydrolase fold common to several other major classes of enzymes, including but not limited to, acetylcholinesterases and carboxylesterases.

5

#### N-acetyltransferase

Aromatic amines and hydrazine-containing compounds are subject to N-acetylation by the N-acetyltransferase enzymes of liver and other tissues. Some xenobiotics can be O-acetylated to some extent by the same enzymes. N-acetyltransferases are cytosolic enzymes which utilize the cofactor acetyl-coenzyme A (acetyl-CoA) to transfer the acetyl group in a two step process. In the first step, the acetyl group is transferred from acetyl-CoA to an active site cysteine residue; in the second step, the acetyl group is transferred to the substrate amino group and the enzyme is regenerated.

In contrast to most other DME classes, there are a limited number of known N-acetyltransferases. In humans, there are two highly similar enzymes, NAT1 and NAT2; mice appear to have a third form of the enzyme, NAT3. The human forms of N-acetyltransferase have independent regulation (NAT1 is widely-expressed, whereas NAT2 is in liver and gut only) and overlapping substrate preferences. Both enzymes appear to accept most substrates to some extent, but NAT1 does prefer some substrates (para-aminobenzoic acid, para-aminosalicylic acid, sulfamethoxazole, and sulfanilamide), while NAT2 prefers others (isoniazid, hydralazine, procainamide, dapsone, aminogluthethimide, and sulfamethazine).

Clinical observations of individuals taking the antituberculosis drug isoniazid in the 1950s led to the description of fast and slow acetylators of the compound. These phenotypes were shown subsequently to be due to mutations in the NAT2 gene which affected enzyme activity or stability. The slow isoniazid acetylator phenotype is very prevalent in Middle Eastern populations (approx. 70%), and is less prevalent in Caucasian (approx. 50%) and Asian (<25%) populations. More recently, functional polymorphism in NAT1 has been detected, with approximately 8% of the population tested showing a slow acetylator phenotype (Butcher, N. J. et al. (1998) Pharmacogenetics 8: 67-72). Since NAT1 can activate some known aromatic amine carcinogens, polymorphism in the widely-expressed NAT1 enzyme may be important in determining cancer risk.

#### Aminotransferases

Aminotransferases comprise a family of pyridoxal 5'-phosphate (PLP)-dependent enzymes that catalyze transformations of amino acids. Aspartate aminotransferase (AspAT) is the most extensively studied PLP-containing enzyme. It catalyzes the reversible transamination of dicarboxylic L-amino acids, aspartate and glutamate, and the corresponding 2-oxo acids, oxalacetate and 2-oxoglutarate. Other members of the family included pyruvate aminotransferase, branched-chain amino acid aminotransferase, tyrosine aminotransferase, aromatic aminotransferase, alanine : glyoxylate aminotransferase (AGT), and kynurenine aminotransferase (Vacca, R. A. et al. (1997) J. Biol. Chem. 272: 21932-21937).



Primary hyperoxaluria type-1 is an autosomal recessive disorder resulting in a deficiency in the liver-specific peroxisomal enzyme, alanine : glyoxylate aminotransferase-1. The phenotype of the disorder is a deficiency in glyoxylate metabolism. In the absence of AGT, glyoxylate is oxidized to oxalate rather than being transaminated to glycine. The result is the deposition of insoluble calcium oxalate in the kidneys and urinary tract, ultimately causing renal failure (Lumb, M. J. et al. (1999) J. Biol. Chem. 274: 20587-20596).

Kynurenine aminotransferase catalyzes the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid. The enzyme may also catalyze the reversible transamination reaction between L-2-aminoadipate and 2-oxoglutarate to produce 2-oxoadipate and L-glutamate. Kynurenic acid is a putative modulator of glutamatergic neurotransmission, thus a deficiency in kynurenine aminotransferase may be associated with pleiotrophic effects (Buchli, R. et al. (1995) J. Biol. Chem. 270: 29330-29335).

#### Catechol-O-Methyltransferase

Catechol-O-methyltransferase (COMT) catalyzes the transfer of the methyl group of S-adenosylmethionine (AdoMet ; SAM) donor to one of the hydroxyl groups of the catechol substrate (e.g., L-dopa, dopamine, or DBA). Methylation of the 3'-hydroxyl group is favored over methylation of the 4'-hydroxyl group and the membrane

bound isoform of COMT is more regiospecific than the soluble form. Translation of the soluble form of the enzyme results from utilization of an internal start codon in a full-length mRNA (1.5 kb) or from the translation of a shorter mRNA (1.3 kb), transcribed from an internal promoter. The proposed  $S_N2$ -like methylation reaction requires  $Mg^{2+}$  and is inhibited by  $Ca^{2+}$ . The binding of the donor and substrate to COMT occurs sequentially. AdoMet first binds COMT in a  $Mg^{2+}$ -independent manner, followed by the binding of  $Mg^{2+}$  and the binding of the catechol substrate.

The amount of COMT in tissues is relatively high compared to the amount of activity normally required, thus inhibition is problematic. Nonetheless, inhibitors have been developed for *in vitro* use (e.g., galates, tropolone, U-0521, and 3',4'-dihydroxy-2-methyl-propionphenetropolone) and for clinical use (e.g., nitrocatechol-based compounds and tolcapone). Administration of these inhibitors results in the increased half-life of L-dopa and the consequent formation of dopamine. Inhibition of COMT is also likely to increase the half-life of various other catechol-structure compounds, including but not limited to epinephrine/norepinephrine, isoprenaline, rimiterol, dobutamine, fenoldopam, apomorphine, and  $\alpha$ -methyldopa. A deficiency in norepinephrine has been linked to clinical depression, hence the use of COMT inhibitors could be useful in the treatment of depression. COMT inhibitors are generally well tolerated with minimal side effects and are ultimately metabolized in the liver with only minor accumulation of metabolites in the body (Männistö, P. T.

and Kaakkola, S. (1999) *Pharmacological Reviews* 51: 593-628).

#### Copper-Zinc Superoxide Dismutases

5        Copper-zinc superoxide dismutases are compact  
homodimeric metalloenzymes involved in cellular defenses  
against oxidative damage. The enzymes contain one atom of  
zinc and one atom of copper per subunit and catalyze the  
10        dismutation of superoxide anions into  $O_2$  and  $H_2O_2$ . The rate  
of dismutation is diffusion-limited and consequently  
enhanced by the presence of favorable electrostatic  
interactions between the substrate and enzyme active site.  
Examples of this class of enzyme have been identified in  
the cytoplasm of all the eukaryotic cells as well as in  
15        the periplasm of several bacterial species. Copper-zinc  
superoxide dismutases are robust enzymes that are highly  
resistant to proteolytic digestion and denaturing by urea  
and SDS. In addition to the compact structure of the  
enzymes, the presence of the metal ions and intrasubunit  
20        disulfide bonds is believed to be responsible for enzyme  
stability. The enzymes undergo reversible denaturation at  
temperatures as high as 70°C (Battistoni, A. et al. (1998)  
*J. Biol. Chem.* 273:655-5661).

Overexpression of superoxide dismutase has been  
25        implicated in enhancing freezing tolerance of transgenic  
Alfalfa as well as providing resistance to environmental  
toxins such as the diphenyl ether herbicide, acifluorfen  
(McKersie, B. D. et al. (1993) *Plant Physiol.* 103: 1155-  
1163). In addition, yeast cells become more resistant to  
30        freeze-thaw damage following exposure to hydrogen peroxide

which causes the yeast cells to adapt to further peroxide stress by upregulating expression of superoxide dismutases. In this study, mutations to yeast superoxide dismutase genes had a more detrimental effect on freeze-thaw resistance than mutations which affected the regulation of glutathione metabolism, long suspected of being important in determining an organism's survival through the process of cryopreservation (Jong-In Park, J-I. et al. (1998) J. Biol. Chem. 273: 22921-22928).

Expression of superoxide dismutase is also associated with *Mycobacterium tuberculosis*, the organism that causes tuberculosis. Superoxide dismutase is one of the ten major proteins secreted by *M. tuberculosis* and its expression is upregulated approximately 5-fold in response to oxidative stress. *M. tuberculosis* expresses almost two orders of magnitude more superoxide dismutase than the nonpathogenic mycobacterium *M. smegmatis*, and secretes a much higher proportion of the expressed enzyme. The result is the secretion of 350-fold more enzyme by *M. tuberculosis* than *M. smegmatis*, providing substantial resistance to oxidative stress (Harth, G. and Horwitz, M. A. (1999) J. Biol. Chem. 274: 4281-4292).

The reduced expression of copper-zinc superoxide dismutases, as well as other enzymes with anti-oxidant capabilities, has been implicated in the early stages of cancer. The expression of copper-zinc superoxide dismutases has been shown to be lower in prostatic intraepithelial neoplasia and prostate carcinomas, compared to normal prostate tissue (Bostwick, D. G. (2000) Cancer 89: 123-134).

## Phosphodiesterases

Phosphodiesterases make up a class of enzymes which catalyze the hydrolysis of one of the two ester bonds in a phosphodiester compound. Phosphodiesterases are therefore  
5 crucial to a variety of cellular processes.

Phosphodiesterases include DNA and RNA endonucleases and exonucleases, which are essential for cell growth and replication, and topoisomerases, which break and rejoin  
10 nucleic acid strands during topological rearrangement of DNA. A Tyr-DNA phosphodiesterase functions in DNA repair by hydrolyzing dead-end covalent intermediates formed between topoisomerase I and DNA (Pouliot, J. J. *et al.* (1999) *Science* 286: 552-555; Yang, S.-W. (1996) *Proc. Natl. Acad. Sci. USA* 93: 11534-11539).

Acid sphingomyelinase is a phosphodiesterase which hydrolyzes the membrane phospholipid sphingomyelin to produce ceramide and phosphorylcholine. Phosphorylcholine is used in the synthesis of phosphatidylcholine, which is  
20 involved in numerous intracellular signaling pathways, while ceramide is an essential precursor for the generation of gangliosides, membrane lipids found in high concentration in neural tissue. Defective acid sphingomyelinase leads to a build-up of sphingomyelin  
25 molecules in lysosomes, resulting in Niemann-Pick disease (Schuchman, E. H. and S. R. Miranda (1997) *Genet. Test.* 1: 13-19).

Glycerophosphoryl diester phosphodiesterase (also known as glycerophosphodiester phosphodiesterase) is a  
30 phosphodiesterase which hydrolyzes deacetylated

phospholipid glycerophosphodiester to produce sn-glycerol-3-phosphate and an alcohol.

Glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoglycerol, and glycerophosphoinositol are examples of substrates for glycerophosphoryl diester phosphodiesterases. A glycerophosphoryl diester phosphodiesterase from *E. coli* has broad specificity for glycerophosphodiester substrates (Larson, T. J. et al. (1983) J. Biol. Chem. 248: 5428-5432).

Cyclic nucleotide phosphodiesterases (PDEs) are crucial enzymes in the regulation of the cyclic nucleotides cAMP and cGMP. cAMP and cGMP function as intracellular second messengers to transduce a variety of extracellular signals including hormones, light, and neurotransmitters. PDEs degrade cyclic nucleotides to their corresponding monophosphates, thereby regulating the intracellular concentrations of cyclic nucleotides and their effects on signal transduction. Due to their roles as regulators of signal transduction, PDEs have been extensively studied as chemotherapeutic targets (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-81; Torphy, J. T. (1998) Am. J. Resp. Crit. CareMed. 157: 351-370).

Families of mammalian PDEs have been classified based on their substrate specificity and affinity, sensitivity to cofactors, and sensitivity to inhibitory agents (Beavo, J. A. (1995) Physiol. Rev. 75: 725-748; Conti, M. et al. (1995) Endocrine Rev. 16: 370-389). Several of these families contain distinct genes, many of which are expressed in different tissues as splice variants. Within

PDE families, there are multiple isozymes and multiple splice variants of these isozymes (Conti, M. and S. L. C. Jin (1999) Prog. Nucleic Acid Res. Mol. Biol. 63: 1-38). The existence of multiple PDE families, isozymes, and splice variants is an indication of the variety and complexity of the regulatory pathways involving cyclic nucleotides (Houslay, M. D. and G. Milligan (1997) Trends Biochem. Sci. 22: 217224).

Type 1 PDEs (PDE1s) are  $\text{Ca}^{2+}$ /calmodulin-dependent and appear to be encoded by at least three different genes, each having at least two different splice variants (Kakkar, R. et al. (1999) Cell Mol. Life Sci. 55: 1164-1186). PDE1s have been found in the lung, heart, and brain. Some PDE1 isozymes are regulated *in vitro* by phosphorylation/dephosphorylation. Phosphorylation of these PDE1 isozymes decreases the affinity of the enzyme for calmodulin, decreases PDE activity, and increases steady state levels of cAMP (Kakkar, *supra*). PDE1s may provide useful therapeutic targets for disorders of the central nervous system, and the cardiovascular and immune systems due to the involvement of PDE1s in both cyclic nucleotide and calcium signaling (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481).

PDE2s are cGMP-stimulated PDEs that have been found in the cerebellum, neocortex, heart, kidney, lung, pulmonary artery, and skeletal muscle (Sadhu, K. et al. (1999) J. Histochem. Cytochem. 47: 895-906). PDE2s are thought to mediate the effects of cAMP on catecholamine secretion, participate in the regulation of aldosterone (Beavo, *supra*), and play a role in olfactory signal transduction

(Juilfs, D. M. et al. (1997) Proc. Natl. Acad. Sci. USA 94: 3388-3395).

PDE3s have high affinity for both cGMP and cAMP, and so these cyclic nucleotides act as competitive substrates for PDE3s. PDE3s play roles in stimulating myocardial contractility, inhibiting platelet aggregation, relaxing vascular and airway smooth muscle, inhibiting proliferation of T-lymphocytes and cultured vascular smooth muscle cells, and regulating catecholamine-induced release of free fatty acids from adipose tissue. The PDE3 family of phosphodiesterases are sensitive to specific inhibitors such as cilostamide, enoximone, and lixazinone. Isozymes of PDE3 can be regulated by cAMP-dependent protein kinase, or by insulin-dependent kinases (Degerman, E. et al. (1997) J. Biol. Chem. 272: 6823-6826).

PDE4s are specific for cAMP, are localized to airway smooth muscle, the vascular endothelium, and all inflammatory cells; and can be activated by cAMP-dependent phosphorylation. Since elevation of cAMP levels can lead to suppression of inflammatory cell activation and to relaxation of bronchial smooth muscle, PDE4s have been studied extensively as possible targets for novel anti-inflammatory agents, with special emphasis placed on the discovery of asthma treatments. PDE4 inhibitors are currently undergoing clinical trials as treatments for asthma, chronic obstructive pulmonary disease, and atopic eczema. All four known isozymes of PDE4 are susceptible to the inhibitor rolipram, a compound which has been shown to improve behavioral memory in mice (Barad, M. et al. (1998) Proc. Natl. Acad. Sci. USA 95: 15020-15025). PDE4



inhibitors have also been studied as possible therapeutic agents against acute lung injury, endotoxemia, rheumatoid arthritis, multiple sclerosis, and various neurological and gastrointestinal indications (Doherty, A. M. (1999)

5 Curr. Opin. Chem. Biol. 3: 466-473).

PDE5 is highly selective for cGMP as a substrate (Turko, I. V. et al. (1998) Biochemistry 37: 4200-4205), and has two allosteric cGMP-specific binding sites (McAllister-Lucas, L. M. et al. (1995) J. Biol. Chem. 270: 10 30671-30679). Binding of cGMP to these allosteric binding sites seems to be important for phosphorylation of PDE5 by cGMP-dependent protein kinase rather than for direct regulation of catalytic activity. High levels of PDE5 are found in vascular smooth muscle, platelets, lung, and 15 kidney. The inhibitor zaprinast is effective against PDE5 and PDE1s. Modification of zaprinast to provide specificity against PDE5 has resulted in sildenafil (VIAGRA; Pfizer, Inc., New York NY), a treatment for male erectile dysfunction (Terrett, N. et al. (1996) Bioorg. 20 Med. Chem. Lett. 6: 1819-1824). Inhibitors of PDE5 are currently being studied as agents for cardiovascular therapy (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481).

PDE6s, the photoreceptor cyclic nucleotide 25 phosphodiesterases, are crucial components of the phototransduction cascade. In association with the G-protein transducin, PDE6s hydrolyze cGMP to regulate cGMP-gated cation channels in photoreceptor membranes. In addition to the cGMP-binding active site, PDE6s also have 30 two high-affinity cGMP-binding sites which are thought to

play a regulatory role in PDE6 function (Artemyev, N. O. et al. (1998) Methods 14: 93-104). Defects in PDE6s have been associated with retinal disease. Retinal degeneration in the rd mouse (Yan, W. et al. (1998) Invest. Ophthalmol. Vis. Sci. 39: 2529-2536), autosomal recessive retinitis pigmentosa in humans (Danciger, M. et al. (1995) Genomics 30: 1-7), and rod/cone dysplasia 1 in Irish Setter dogs (Suber, M. L. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 3968- 972) have been attributed to mutations in the PDE6B gene.

The PDE7 family of PDEs consists of only one known member having multiple splice variants (Bloom, T. J. and J. A. Beavo (1996) Proc. Natl. Acad. Sci. USA 93: 14188-14192). PDE7s are cAMP specific, but little else is known about their physiological function. Although mRNAs encoding PDE7s are found in skeletal muscle, heart, brain, lung, kidney, and pancreas, expression of PDE7 proteins is restricted to specific tissue types (Han, P. et al. (1997) J. Biol. Chem. 272: 16152-16157 ; Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481). PDE7s are very closely related to the PDE4 family; however, PDE7s are not inhibited by rolipram, a specific inhibitor of PDE4s (Beavo, *supra*).

PDE8s are cAMP specific, and are closely related to the PDE4 family. PDE8s are expressed in thyroid gland, testis, eye, liver, skeletal muscle, heart, kidney, ovary, and brain. The cAMP hydrolyzing activity of PDE8s is not inhibited by the PDE inhibitors rolipram, vinpocetine, milrinone, IBMX (3-isobutyl-1-methylxanthine), or zaprinast, but PDE8s are inhibited by dipyrindamole

(Fisher, D. A. et al. (1998) Biochem. Biophys. Res. Commun. 246: 570-577 ; Hayashi, M. et al. (1998) Biochem. Biophys. Res. Commun. 250: 751-756 ; Soderling, S. H. et al. 1998) Proc. Natl. Acad. Sci. USA 95: 8991-8996).

5 PDE9s are cGMP specific and most closely resemble the PDE8 family of PDEs. PDE9s are expressed in kidney, liver, lung, brain, spleen, and small intestine. PDE9s are not inhibited by sildenafil (VIAGRA; Pfizer, Inc., New York NY), rolipram, vinpocetine, dipyridamole, or IBMX (3-  
10 isobutyl-lmethylxanthine), but they are sensitive to the PDE5 inhibitor zaprinast (Fisher, D. A. et al. (1998) J. Biol. Chem. 273: 15559-15564 ; Soderling, S. H. et al. (1998) J. Biol. Chem. 273: 15553-15558).

PDE10s are dual-substrate PDEs, hydrolyzing both cAMP  
15 and cGMP. PDE10s are expressed in brain, thyroid, and testis. (Soderling, S. H. et al. (1999) Proc. Natl. Acad. Sci. USA 96: 7071-7076; Fujishige, K. et al. (1999) J. Biol. Chem. 274: 18438-18445; Loughney, K. et al. (1999) Gene 234: 109117).

20 PDEs are composed of a catalytic domain of about 270-300 amino acids, an N-terminal regulatory domain responsible for binding cofactors, and, in some cases, a hydrophilic C-terminal domain of unknown function (Conti, M. and S.-L. C. Jin (1999) Prog. Nucleic Acid Res. Mol. Biol. 63: 1-  
25 38). A conserved, putative zinc-binding motif, HDXXHXGXXN, has been identified in the catalytic domain of all PDEs. N-terminal regulatory domains include non-catalytic cGMP-binding domains in PDE2s, PDE5s, and PDE6s; calmodulin-binding domains in PDE1s; and domains containing  
30 phosphorylation sites in PDE3s and PDE4s. In PDE5, the N-

terminal cGMP-binding domain spans about 380 amino acid residues and comprises tandem repeats of the conserved sequence motif N (R/K) XnFX3DE (McAllister-Lucas, L. M. et al. (1993) J. Biol. Chem. 268: 22863-22873). The NKXnD motif has been shown by mutagenesis to be important for cGMP binding (Turko, I. V. et al. (1996) J. Biol. Chem. 271: 22240-22244). PDE families display approximately 30% amino acid identity within the catalytic domain; however, isozymes within the same family typically display about 85-95% identity in this region (e.g. PDE4A vs PDE4B). Furthermore, within a family there is extensive similarity (>60%) outside the catalytic domain; while across families, there is little or no sequence similarity outside this domain.

Many of the constituent functions of immune and inflammatory responses are inhibited by agents that increase intracellular levels of cAMP (Verghese, M. W. et al. (1995) Mol. Pharmacol. 47: 1164-1171). A variety of diseases have been attributed to increased PDE activity and associated with decreased levels of cyclic nucleotides. For example, a form of diabetes insipidus in mice has been associated with increased PDE4 activity, an increase in low- $K_m$  cAMP PDE activity has been reported in leukocytes of atopic patients, and PDE3 has been associated with cardiac disease.

Many inhibitors of PDEs have been identified and have undergone clinical evaluation (Perry, M. J. and G. A. Higgs (1998) Curr. Opin. Chem. Biol. 2: 472-481; Torphy, T. J. (1998) Am. J. Respir. Crit. Care Med. 157: 351-370). PDE3 inhibitors are being developed as

antithrombotic agents, antihypertensive agents, and as  
cardiotonic agents useful in the treatment of congestive  
heart failure. Rolipram, a PDE4 inhibitor, has been used  
in the treatment of depression, and other inhibitors of  
5 PDE4 are undergoing evaluation as anti-inflammatory  
agents. Rolipram has also been shown to inhibit  
lipopolysaccharide (LPS) induced TNF-a, which has been  
shown to enhance HIV-1 replication in vitro. Therefore,  
rolipram may inhibit HIV-1 replication (Angel, J. B. et  
10 al. (1995) AIDS 9: 1137-1144). Additionally, rolipram,  
based on its ability to suppress the production of  
cytokines such as TNF-a and b and interferon g, has been  
shown to be effective in the treatment of  
encephalomyelitis. Rolipram may also be effective in  
15 treating tardive dyskinesia and was effective in treating  
multiple sclerosis in an experimental animal model  
(Sommer, N. et al. (1995) Nat. Med. 1: 244-248; Sasaki, H.  
et al. (1995) Eur. J. Pharmacol. 282: 71-76).

Theophylline is a nonspecific PDE inhibitor used in  
20 the treatment of bronchial asthma and other respiratory  
diseases. Theophylline is believed to act on airway smooth  
muscle function and in an anti-inflammatory or  
immunomodulatory capacity in the treatment of respiratory  
diseases (Banner, K. H. and C. P. Page (1995) Eur. Respir.  
25 J. 8: 996-1000). Pentoxifylline is another nonspecific PDE  
inhibitor used in the treatment of intermittent  
claudication and diabetes-induced peripheral vascular  
disease. Pentoxifylline is also known to block TNF-a  
production and may inhibit HIV-1 replication (Angel et  
30 al., *supra*).

PDEs have been reported to affect cellular proliferation of a variety of cell types (Conti et al. (1995) Endocrine Rev. 16: 370-389) and have been implicated in various cancers. Growth of prostate carcinoma cell lines DU145 and LNCaP was inhibited by delivery of cAMP derivatives and PDE inhibitors (Bang, Y. J. et al. (1994) Proc. Natl. Acad. Sci. USA 91: 5330-5334). These cells also showed a permanent conversion in phenotype from epithelial to neuronal morphology. It has also been suggested that PDE inhibitors have the potential to regulate mesangial cell proliferation (Matousovic, K. et al. (1995) J. Clin. Invest. 96: 401-410) and lymphocyte proliferation (Joulain, C. et al. (1995) J. Lipid Mediat. Cell Signal. 11: 63-79). A cancer treatment has been described that involves intracellular delivery of PDEs to particular cellular compartments of tumors, resulting in cell death (Deonarain, M. P. and A. A. Epenetos (1994) Br. J. Cancer 70: 786-794).

## 20 Phosphotriesterases

Phosphotriesterases (PTE, paraoxonases) are enzymes that hydrolyze toxic organophosphorus compounds and have been isolated from a variety of tissues. The enzymes appear to be lacking in birds and insects, but is abundant in mammals, explaining the reduced tolerance of birds and insects to organophosphorus compound (Vilanova, E. and Sogorb, M. A. (1999) Crit. Rev. Toxicol. 29: 21-57).

Phosphotriesterases play a central role in the detoxification of insecticides by mammals.

30 Phosphotriesterase activity varies among individuals and

is lower in infants than adults. Knockout mice are markedly more sensitive to the organophosphate-based toxins diazoxon and chlorpyrifos oxon (Furlong, C. E., et al. (2000) Neurotoxicology 21: 91-100). PTEs have  
5 attracted interest as enzymes capable of the detoxification of organophosphate-containing chemical waste and warfare reagents (e.g., parathion), in addition to pesticides and insecticides. Some studies have also implicated phosphotriesterase in atherosclerosis and  
10 diseases involving lipoprotein metabolism.

#### Thioesterases

Two soluble thioesterases involved in fatty acid biosynthesis have been isolated from mammalian tissues,  
15 one which is active only toward long-chain fatty-acyl thioesters and one which is active toward thioester with a wide range of fatty-acyl chain-lengths. These thioesterases catalyze the chain-terminating step in the de novo biosynthesis of fatty acids. Chain termination  
20 involves the hydrolysis of the thioester bond which links the fatty acyl chain to the 4'-phosphopantetheine prosthetic group of the acyl carrier protein (ACP) subunit of the fatty acid synthase (Smith, S. (1981a) Methods Enzymol. 71: 181-188; Smith, S. (1981b) Methods Enzymol.  
25 71: 188-200).

*E. coli* contains two soluble thioesterases, thioesterase I (TEI) which is active only toward long-chain acyl thioesters, and thioesterase II (TEII) which has a broad chain-length specificity (Naggert, J. et al.  
30 (1991) J. Biol. Chem. 266: 11044-11050). *E. coli* TEII does

not exhibit sequence similarity with either of the two types of mammalian thioesterases which function as chain-terminating enzymes in *de novo* fatty acid biosynthesis. Unlike the mammalian thioesterases, *E. coli* TEII lacks the characteristic serine active site gly-X-ser-X-gly sequence motif and is not inactivated by the serine modifying agent diisopropyl fluorophosphate. However, modification of histidine 58 by iodoacetamide and diethylpyrocarbonate abolished TEII activity. Overexpression of TEII did not alter fatty acid content in *E. coli*, which suggests that it does not function as a chain-terminating enzyme in fatty acid biosynthesis (Naggert et al., *supra*). For that reason, Naggert et al. (*supra*) proposed that the physiological substrates for *E. coli* TEII may be coenzyme A (CoA)-fatty acid esters instead of ACP-phosphopanthetheine-fatty acid esters.

#### Carboxylesterases

Mammalian carboxylesterases constitute a multigene family expressed in a variety of tissues and cell types. Isozymes have significant sequence homology and are classified primarily on the basis of amino acid sequence. Acetylcholinesterase, butyrylcholinesterase, and carboxylesterase are grouped into the serine super family of esterases (B-esterases). Other carboxylesterases included thyroglobulin, thrombin, Factor IX, gliotactin, and plasminogen. Carboxylesterases catalyze the hydrolysis of ester and amide-groups from molecules and are involved in detoxification of drugs, environmental toxins, and carcinogens. Substrates for carboxylesterases include



short-and long-chain acyl-glycerols, acylcarnitine, carbonates, dipivefrin hydrochloride, cocaine, salicylates, capsaicin, palmitoyl-coenzyme A, imidapril, haloperidol, pyrrolizidine alkaloids, steroids, p-nitrophenyl acetate, malathion, butanilicaine, and isocarboxazide. The enzymes often demonstrate low substrate specificity. Carboxylesterases are also important for the conversion of prodrugs to their respective free acids, which may be the active form of the drug (e.g., lovastatin, used to lower blood cholesterol) (reviewed in Satoh, T. and Hosokawa, M. (1998) *Annu. Rev. Pharmacol. Toxicol.* 38: 257-288).

Neuroligins are a class of molecules that (i) have N-terminal signal sequences, (ii) resemble cell surface receptors, (iii) contain carboxylesterase domains, (iv) are highly expressed in the brain, and (v) bind to neurexins in a calcium-dependent manner. Despite the homology to carboxylesterases, neuroligins lack the active site serine residue, implying a role in substrate binding rather than catalysis (Ichtchenko, K. et al. (1996) *J. Biol. Chem.* 271: 2676-2682).

#### Squalene Epoxidase

Squalene epoxidase (squalene monooxygenase, SE) is a microsomal membrane-bound, FAD-dependent oxidoreductase that catalyzes the first oxygenation step in the sterol biosynthetic pathway of eukaryotic cells. Cholesterol is an essential structural component of cytoplasmic membranes acquired via the LDL receptor-mediated pathway or the biosynthetic pathway. In the latter case, all 27 carbon

atoms in the cholesterol molecule are derived from acetyl-CoA (Stryer, L., *supra*). SE converts squalene to 2, 3 (S)-oxidosqualene, which is then converted to lanosterol and then cholesterol. The steps involved in cholesterol

5 biosynthesis are summarized below (Stryer, L (1988) Biochemistry. W. H Freeman and Co., Inc. New York. pp. 554-560 and Sakakibara, J. et al. (1995) 270: 17-20) :  
acetate (from Acetyl-CoA) 3-hydroxy-3-methyl-glutaryl CoA  
mevalonate 5-phosphomevalonate 5-  
10 pyrophosphomevalonate isopentenyl pyrophosphate  
dimethylallyl pyrophosphate geranyl pyrophosphate  
farnesyl pyrophosphate squalene squalene epoxide  
lanosterol cholesterol.

While cholesterol is essential for the viability of  
15 eukaryotic cells, inordinately high serum cholesterol  
levels results in the formation of atherosclerotic plaques  
in the arteries of higher organisms. This deposition of  
highly insoluble lipid material onto the walls of  
essential blood vessels (e.g., coronary arteries) results  
20 in decreased blood flow and potential necrosis of the  
tissues deprived of adequate blood flow. HMG-CoA reductase  
is responsible for the conversion of 3-hydroxyl-3-  
methylglutaryl CoA (HMG-CoA) to mevalonate, which  
represents the first committed step in cholesterol  
25 biosynthesis. HMG-CoA is the target of a number of  
pharmaceutical compounds designed to lower plasma  
cholesterol levels. However, inhibition of MHG-CoA also  
results in the reduced synthesis of non-sterol  
intermediates (e.g., mevalonate) required for other  
30 biochemical pathways. SE catalyzes a rate-limiting

reaction that occurs later in the sterol synthesis pathway and cholesterol is the only end product of the pathway following the step catalyzed by SE. As a result, SE is the ideal target for the design of anti-hyperlipidemic drugs that do not cause a reduction in other necessary intermediates (Nakamura, Y. et al. (1996) 271: 8053-8056).

### Epoxide hydrolases

Epoxide hydrolases catalyze the addition of water to epoxide-containing compounds, thereby hydrolyzing epoxides to their corresponding 1, 2-diols. They are related to bacterial haloalkane dehalogenases and show sequence similarity to other members of the  $\alpha/\beta$  hydrolase fold family of enzymes (e.g., bromoperoxidase A2 from *Streptomyces aureofaciens*, hydroxymuconic semialdehyde hydrolases from *Pseudomonas putida*, and haloalkane dehalogenase from *Xanthobacter autotrophicus*). Epoxide hydrolases are ubiquitous in nature and have been found in mammals, invertebrates, plants, fungi, and bacteria. This family of enzymes is important for the detoxification of xenobiotic epoxide compounds which are often highly electrophilic and destructive when introduced into an organism. Examples of epoxide hydrolase reactions include the hydrolysis of cis-9, 10-epoxyoctadec-9 (Z)-enoic acid (leukotoxin) to form its corresponding diol, threo-9, 10-dihydroxyoctadec-12 (Z)-enoic acid (leukotoxin diol), and the hydrolysis of cis-12, 13-epoxyoctadec-9 (Z)-enoic acid (isoleukotoxin) to form its corresponding diol threo-12, 13-dihydroxyoctadec-9 (Z)-enoic acid (isoleukotoxin diol). Leukotoxins alter membrane permeability and ion transport

and cause inflammatory responses. In addition, epoxide carcinogens are known to be produced by cytochrome P450 as intermediates in the detoxification of drugs and environmental toxins.

5       The enzymes possess a catalytic triad composed of Asp (the nucleophile), Asp (the histidine-supporting acid), and His (the water-activating histidine). The reaction mechanism of epoxide hydrolase proceeds via a covalently bound ester intermediate initiated by the nucleophilic  
10       attack of one of the Asp residues on the primary carbon atom of the epoxide ring of the target molecule, leading to a covalently bound ester intermediate (Michael Arand, M. et al. (1996) J. Biol. Chem. 271: 4223-4229; Rink, R. et al. (1997) J. Biol. Chem. 272: 14650-14657; Argiriadi,  
15       M. A. et al. (2000) J. Biol. Chem. 275: 15265-15270).

#### Enzymes involved in tyrosine catalysis

      The degradation of the amino acid tyrosine to either succinate and pyruvate or fumarate and acetoacetate,  
20       requires a large number of enzymes and generates a large number of intermediate compounds. In addition, many xenobiotic compounds may be metabolized using one or more reactions that are part of the tyrosine catabolic pathway. While the pathway has been studied primarily in bacteria,  
25       tyrosine degradation is known to occur in a variety of organisms and is likely to involve many of the same biological reactions.

      The enzymes involved in the degradation of tyrosine to succinate and pyruvate (e.g., in *Artlirobacter* species)  
30       include 4-hydroxyphenylpyruvate oxidase, 4-

hydroxyphenylacetate 3-hydroxylase, 3, 4-dihydroxyphenylacetate 2, 3-dioxygenase, 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase, trans, cis-5-carboxymethyl-2-hydroxymuconate isomerase, 5 homoprotocatechuate isomerase/decarboxylase, cis-2-oxohept-3-ene-1, 7-dioate hydratase, 2, 4-dihydroxyhept-trans-2-ene-1, 7-dioate aldolase, and succinic semialdehyde dehydrogenase.

The enzymes involved in the degradation of tyrosine to 10 fumarate and acetoacetate (e.g., in *Pseudomonas* species) include 4-hydroxyphenylpyruvate dioxygenase, homogentisate 1, 2-dioxygenase, maleylacetoacetate isomerase, and fumarylacetoacetase. 4-hydroxyphenylacetate 1-hydroxylase may also be involved if intermediates from the 15 succinate/pyruvate pathway are accepted.

Additional enzymes associated with tyrosine metabolism in different organisms include 4-chlorophenylacetate-3, 4-dioxygenase, aromatic aminotransferase, 5-oxopent-3-ene-1, 2, 5-tricarboxylate decarboxylase, 2-oxo-hept-3-ene-1, 7- 20 dioate hydratase, and 5-carboxymethyl-2-hydroxymuconate isomerase (Ellis, L. B. M. et al. (1999) *Nucleic Acids Res.* 27: 373-376; Wackett, L. P. and Ellis, L. B. M. (1996) *J. Microbiol. Meth.* 25: 91-93; and Schmidt, M. (1996) *Amer. Soc. Microbiol. News* 62: 102).

25 In humans, acquired or inherited genetic defects in enzymes of the tyrosine degradation pathway may result in hereditary tyrosinemia. One form of this disease, hereditary tyrosinemia 1 (HT1) is caused by a deficiency in the enzyme fumarylacetoacetate hydrolase, the last 30 enzyme in the pathway in organisms that metabolize

tyrosine to fumarate and acetoacetate. HT1 is characterized by progressive liver damage beginning at infancy, and increased risk for liver cancer (Endo, F. et al. (1997) J. Biol. Chem. 272: 24426-24432).

5       An enzyme of one system can act on several drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic polymorphism within each system. Metabolic phenotypes have been generally  
10       characterized as poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM). Knowledge of a metabolic phenotype is clinically useful for the following reasons:

15       a)     a phenotype may be correlated to an individual's susceptibility to toxic chemicals, diseases and cancers;

      b)     a phenotype may provide a physician with valuable information for quickly determining a safe and therapeutically-effective drug treatment regimen for an individual; and

20       c)     individual phenotypes may provide valuable rationales for the design of therapeutic drugs.

      To date, the ability to characterize multiple phenotypic determinants for the purpose of identifying individual phenotypes, drug treatment compatibility and  
25       susceptibility has been limited by the complexities of multiple metabolic pathways, and the lack of efficient and effective procedures for making these determinations. Currently, the determination of an individual's phenotype for a given metabolic enzyme can be performed either via

direct metabolic phenotyping or indirect extrapolation of an individual's genotype to a given phenotype.

Direct phenotyping involves the use a probe substrate known to be metabolized by a given enzyme. The rate of metabolism of the probe substrate is measured and this rate of metabolism is used to determine a metabolic phenotype. Although labor intensive and costly procedures for direct phenotyping have been known for many years these procedures are not readily adaptable for a clinical environment, nor are they practical for measuring multiple phenotypic determinants. For example, enzymatic phenotypes may be determined by measurements of the molar (or chiral) ratio of metabolites of a drug or a probe substrate in a urine sample from a individual by high-pressure liquid chromatography (HPLC), capillary electrophoresis (CE) or stereo-selective capillary gas chromatography. These determination methods are time-consuming, onerous, and employ systems and equipment that are not readily available in a clinical laboratory. Methodologies for the rapid determination of multiple determinants of a metabolic phenotypic are not available, and as a result, valuable information concerning an individual's phenotype is not considered on a routine basis in a clinical environment.

Indirect phenotyping can be defined as assigning a phenotype based on non-functional measurements. These non-functional measurements include genotyping, haplotyping, gene expression and protein expression analysis. The patent application, WO 00/63683 provides an extensive

description of various methods developed to perform the  
aforementioned analysis.

Genotyping is performed by analyzing the genetic  
sequence of a gene coding for a specific enzyme by a  
5 polymerase chain reaction assay (PCR) or a PCR with a  
restriction fragment length polymorphism assay (PCR-RFLP).  
The gene is examined for the presence of genetic mutations  
that can be linked to increased or decreased enzyme levels  
or activity, which in turn result in a specific phenotype,  
10 i.e. a slow metabolizer vs. a fast metabolizer. The  
genotype is a theoretical measurement of what an  
individual's phenotype should be. Haplotyping is an  
extension of genotyping in which the genotype of different  
gene alleles are considered. For example if a person had  
15 one wild type (wt) gene sequence and one mutant (mt) gene  
sequence, the individual would have a wt/mt haplotype.  
Gene expression and protein expression analysis is defined  
as the measurement of mRNA/cDNA and protein levels  
respectively.

20 Indirect phenotyping may be limited by several factors  
that can result in an alteration in the theoretical  
phenotype. For example it has been well established that  
genotype does not always correlate with phenotype,  
likewise gene expression does not always correlate with  
25 protein expression, and protein expression does not always  
correlate with protein function. Indirect phenotyping  
fails to account for many factors that affect protein  
function including but not limited to post-translational  
protein modification, polypharmacy, and exposure to  
30 inducers or inhibitors. Furthermore, other limitations



include the potential complexity of performing a complete genotyping. The mutation sequence must first be identified before they can be examined in a genotyping assay.

Subsequent to identification, the mutation must be linked  
5 to a definitive effect on phenotype. For some enzymes, there appear to be very few mutations and those found have been well characterized, while for other enzymes multiple mutations are present with new mutations being found regularly (e.g. CYP2D6 has over 53 mutations and 48  
10 allelic variants). Therefore, while genotyping for CYP2C19 might be performed with relatively few measurements, a complete and accurate genotyping of CYP2D6 would be complex and require multiple measurements.

Indirect phenotyping suffers from complexity and the  
15 direct phenotyping techniques are not easily accessible to clinical settings,

Physicians routinely prescribe treatment regimes without knowledge of an individual's metabolic capability (phenotype) or genotype for metabolism. Accordingly, a  
20 trial and error treatment regime is initiated, often at the expense of severe side effects and loss of valuable treatment time.

The need for a method to predict an individual's response to a drug therapy (both efficacy of therapy and  
25 occurrence of side effects) has been recognized by many in the field. In the following section a brief description of the current technologies and applications will be described. The description of these technologies and applications should in no way be taken as a recognition of  
30 these technologies and applications as prior art but

rather as a means of differentiating the current invention from these technologies and applications.

5 Rosetta Inpharmatics describes a method of analyzing biological response and toxicity by monitoring what they described as target pathways (response) and off-target pathways (toxicity) (US5, 965, 352; WO99/58708; WO00/39337; WO00/39340; US6222093 B1). The monitoring of these pathways is limited to an analysis of gene or protein expression data. They perform this analysis in pre and post drug treatment samples. These patents make no claims to individualizing the dose of drug to be given to an individual. A similar position is described by Zimmer (EP 1 158 447 A15). Zimmer claims the analysis of biological systems, including metabolic (organic  
10 molecules) pathways. However, this application again measures endogenous products without a probe substrate and makes claims for drug development, target validation and disease diagnosis, but not for individualizing drug doses.

A similar patent application, Phase I Molecular  
20 Toxicology (PCT/US00/30474), describes a method of determining individual hypersensitivity to a given drug therapy through the analysis of gene or protein expression data. The expression profile of a hypersensitive individual is compared to the expression profile of an individual who is not hypersensitive. A differential  
25 pattern is isolated which correlates with the hypersensitivity. This pattern is then used to identify other individuals who would be hypersensitive if exposed to the drug. This patent application makes no claims to  
30 individualizing drug dosing.

Recognizing the limitations of genotyping or expression analysis, others have attempted to predict response and/or toxicity through an analysis of the biochemical make up of the whole body or of individual cells. The hypothesis is that pathophysiological perturbations result in disturbances in the ratios, concentrations, bindings or fluxes of endogenous biochemicals, either by direct chemical reactions or by binding to key enzymes or nucleic acids that control metabolism. This field has been defined as metabonomics or metomics and is described by Lindon et al. (Concepts in Magnetic Resonance 12(5)289-320, 2000). Various methods of performing this analysis have been described (WO01/78652 A2; US6087090; US5824467; US6210970; US6194217; WO00/65472, WO00/63683). None of these positions claim individualization of drug dose levels. Additionally, all of these patents (with the exception of WO 00/63683) claim the analysis of endogenous biochemicals or metabolites, rather than those of an exogenous probe substrate.

Other methods describe methods of determining enzyme function. For example, Henry et al. (US6113763) describe a method of measuring the effect of a compound on various enzyme systems. However, this patent is limited to *in vitro* analysis after treatment of mammalian cell cultures.

Many of the preceding technologies and applications claim to be able to allow the classification of individuals as responders and non-responders, or classification as likely to suffer toxicity or those that can safely take the drug. Unfortunately, none of these technologies and applications take into account variations

in drug metabolism. The importance of drug metabolizing can be explained as follows. If inhibition of a particular system leads to toxicity, then low gene or protein expression of components of this system might be used to  
5 identify individuals with high risk of toxicity. Likewise those individual's with high expression levels would be considered to be at low risk. However, if the individual classified as a low risk subject, also has low metabolism of the drug, then the drug will remain in the system much  
10 longer and may have the time to eliminate the function of the system which as a result leads to toxicity. Conversely, if an individual has low system activity but is also a rapid drug metabolizer, than it is possible that there will not be sufficient drug present at any given  
15 point to induce toxicity by inhibiting the system. Therefore, the knowledge of an individual's drug metabolizing capabilities is an essential component of individualized drug therapy.

The ability to rapidly and accurately identify  
20 multiple metabolic phenotypic determinants on an individual basis would provide a physician with valuable individual-specific information that could be readily applied in selecting a safe and effective treatment regime for that individual. Similarly, knowledge of multi-  
25 determinant metabolic phenotypics would also find valuable application in research and drug development. In particular, individual phenotypes could be identified prior to a drug treatment trial. Moreover, knowledge of multi-determinant metabolic phenotypes would have

applications in the development of new drugs, so-called rational drug design.

#### SUMMARY OF THE INVENTION

5        One aim of the present invention is to provide methods for multi-determinant metabolic phenotyping.

Another aim of the invention is to provide a method for determining enzyme-specific phenotypic determinants of an individual using a non-toxic probe substrate(s) to  
10       predict his/her predisposed response to a wide range of potentially toxic drugs. In doing so, the present invention could be readily employed to screen individuals for their compatibility with an identified drug treatment. This would, provide valuable information about that  
15       individual's ability to metabolize the identified drug and predict the likelihood of a toxic response thereto.

Accordingly, another aim of the present invention is to provide a method for selecting an individual treatment regime.

20       Yet another aim of the present invention is to provide a method for selecting an individual drug treatment regime using genotyping.

Yet another aim of the present invention is to provide a method for selecting candidates for clinical treatment  
25       trials.

Still another aim of the present invention is to provide a method of using multi-determinant phenotyping for rational drug design and development.

In accordance with one aspect of the invention, there is provided a method of characterizing a multi-determinant

metabolic phenotype, wherein a plurality of phenotypic determinants are identified as corresponding to respective metabolic characteristics, said method comprising: administering to an individual a probe substrate specific to a metabolic pathway(s) of each of said plurality of phenotypic determinants; detecting metabolites of said metabolic pathway(s) in a biological sample from said individual in response to said probe substrate; and characterizing respective phenotypic determinants of said multi-determinant metabolic phenotype based thereon.

In accordance with another aspect of the invention, there is provided a method of characterizing a multi-determinant metabolic phenotype of an individual, said method comprising: administering a plurality of probe  
5 substrates to said individual; each probe substrate being specific to at least one metabolic pathway of interest; determining respective amounts of determinant-specific metabolites in a biological sample obtained from said individual at a time after said plurality of probe  
10 substrates has been consumed; calculating ratios of said metabolites for said respective determinants; and characterizing said multi-determinant metabolic phenotype from said ratios, wherein each determinant corresponds to a phenotypic trait of the respective metabolic pathways of  
15 interest.

In accordance with a further aspect of the invention, there is provided a method of using a multi-determinant metabolic phenotype to select a drug treatment regimen for an individual, said method comprising: comparing a  
20 metabolic profile of a candidate drug with said multi-

determinant metabolic phenotype of said individual, and if said multi-determinant metabolic phenotypic is indicative of a phenotype having metabolic efficiency for said candidate drug, selecting said candidate drug for use in the treatment regimen for said individual.

In accordance with yet another aspect of the invention, there is provided a method of using a multi-determinant metabolic phenotype to individualize a selected drug treatment regimen for an individual, wherein the multi-determinant metabolic phenotype of said individual is determined; a therapeutically effective dose of said drug treatment is determined based on said multi-determinant phenotype; and selecting said therapeutically effective dose for use in the treatment regimen for said individual.

In accordance with yet a further aspect of the invention, there is provided a method of using a multi-determinant metabolic phenotype to individualize the drug treatment regimen of a class or genus of compounds with similar metabolic profiles, wherein the multi-determinant metabolic phenotype of an individual is determined and a therapeutically effective dose of said class or genus of compounds is determined on the basis of said multi-determinant phenotype of said individual and said dose is selected for use in the treatment regimen for said individual.

In accordance with another aspect of the invention, there is provided a method of treating a individual with a medical condition with a therapeutically effective drug treatment known for use with said condition, said method

comprising: administering at least one non-toxic probe substrate representative of at least one compound known for treating said condition to said individual; obtaining a biological sample from said individual at a  
5 predetermined time after administering the at least one probe substrate; detecting determinant-specific metabolites in said biological sample; determining phenotypic determinants indicative of the individual's ability to metabolize said at least one compound in said  
10 group of compounds; and administering a therapeutically effective amount of at least one compound having a metabolic pattern corresponding to said individual's metabolic phenotype as represented by said phenotypic determinant.

15 In accordance with a further aspect of the invention, there is provided a method of selecting a treatment for an individual corresponding to said individual's metabolic phenotype, said method comprising: identifying a group of candidate treatments; administering at least one probe  
20 substrate representative of each of said candidate treatments to said individual; determining phenotypic determinants for said at least one probe substrate from a biological sample obtained from said individual at a time after administering the probe substrate; and identifying a  
25 treatment from said group of treatments that corresponds to said individual's enzyme-specific capacity according to said phenotypic determinants; and selecting the same.

In accordance with yet a further aspect of the invention, there is provided a method of screening  
30 individuals for participation in a drug treatment trial



assessing the therapeutic effect of a drug, said method comprising: administering a non-toxic probe substrate having metabolic characteristics corresponding to said candidate drug to said individuals; measuring for

5 phenotypic determinants indicative of metabolic efficiency for the metabolism of said probe substrate in a biological sample obtained from said individuals; characterizing the metabolic phenotypes of said plurality of individuals; and identifying those individuals having a metabolic phenotype

10 characterized as effective for metabolizing said candidate drug.

The invention also provides a method of selectively treating individuals suffering from disease X with a known drug Y for treatment thereof, said method comprising:

15 detecting determinant-specific metabolites in a biological sample of an individual suffering from disease X after treatment with a probe substrate corresponding to drug Y; characterizing a phenotypic determinant based on amounts of said determinant-specific metabolites; and determining

20 if said phenotypic determinant qualifies as a metabolic phenotype known to effectively metabolize drug Y; and administering drug Y if the metabolic phenotype of an individual is known to effectively metabolize drug Y.

The invention further provides an assay system for

25 detecting the presence of multiple determinant-specific metabolites in a biological sample obtained from an individual treated with at least one stimulus specific for metabolic pathways of said metabolites; said system comprising: means for receiving said biological sample,

30 including a plurality of affinity complexation agents

contained therein; means for detecting the presence of  
said metabolites bound to said affinity complexation  
agents; and means for quantifying molar ratios of said  
metabolites to provide corresponding phenotypic  
5 determinants; wherein said phenotypic determinants provide  
a metabolic phenotype profile.

The invention yet further provides a method of  
identifying a therapeutically effective drug treatment  
dosage for an individual, said method comprising:  
10 determining an enzyme-specific genotype of said individual  
corresponding to the metabolism of a candidate drug; and  
identifying a therapeutically effective amount of said  
drug for treating said individual based on said genotype.

For the purpose of the present invention the following  
15 terms are defined below.

The term "phenotypic determinant" is intended to mean  
a qualitative or quantitative indicator of an enzyme-  
specific capacity of an individual.

The term "biological sample" is intended to mean a  
20 sample obtained from a biological entity and includes, but  
is not to be limited to, any one of the following: tissue,  
cerebrospinal fluid, plasma, serum, saliva, blood, nasal  
mucosa, urine, synovial fluid, microcapillary  
microdialysis and breath.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates metabolites of the CYP3A4 enzymatic  
pathway according to an embodiment of the present  
invention;

Fig. 2 illustrates metabolites of the NAT1 enzymatic pathway according to an embodiment of the present invention;

5 Fig. 3 illustrates metabolites of the CYP1A2 enzymatic pathway according to another embodiment of the present invention;

Fig. 4 illustrates metabolites of the CYP2A6 enzymatic pathway according to another embodiment of the present invention;

10 Fig. 5 illustrates metabolites of the CYP2C19 enzymatic pathway according to another embodiment of the present invention;

15 Fig. 6 illustrates metabolites of the CYP2C9 enzymatic pathway according to another embodiment of the present invention;

Fig. 7 illustrates metabolites of the CYP2D6 enzymatic pathway according to another embodiment of the present invention;

20 Fig. 8 illustrates metabolites of the CYP2E1 enzymatic pathway according to another embodiment of the present invention;

Fig. 9 illustrates metabolites of the NAT2 enzymatic pathway according to another embodiment of the present invention;

25 Fig. 10 illustrates the scheme of the general immunosensor design depicting the intimate integration of immunological recognition at the solid-state surface and the signal transduction;

Fig. 11 illustrates the principle of SPR technology;

30 Fig. 12 illustrates a QCM immunosensor device;

Fig. 13 illustrates the synthetic routes for the production of AAMU and 1X derivatives used in accordance with one embodiment of the present invention;

5 Figs. 14 to 17 show other AAMU and 1X derivatives which can be used for raising antibodies in accordance with another embodiment of the present invention;

Fig. 18 illustrates the absorbance competitive antigen ELISA curves of AAMU-Ab and 1X-Ab in accordance with one embodiment of the present invention;

10 Fig. 19 is a histogram of molar ratio of AAMU/1X;

Fig. 20 illustrates the synthetic routes for the production of caffeine and 1,7-dimethylxanthine derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

15 Fig. 21 illustrates the synthetic routes for the production of caffeine and 1,7-dimethyluric acid derivatives for CYP1A2 phenotyping in accordance with one embodiment of the present invention;

20 Fig. 22 illustrates an array of microwell plates as employed in accordance with another embodiment of the present invention;

Fig. 23 illustrates an ELISA array in accordance with an embodiment of the present invention;

25 Fig. 24 illustrates an example of the configuration of a microwell plate in accordance with another embodiment of the present invention;

Fig. 25 illustrates a rapid immunoassay system in accordance with another embodiment of the present invention;

Fig. 26 illustrates an ELISA detection system in accordance with another embodiment of the present invention;

5 Fig. 27 illustrates a rapid immunoassay system in accordance with yet another embodiment of the present invention;

Fig. 28 illustrates a PCR ELISA schematic in accordance with an embodiment of the present invention; and

10 Fig. 29 illustrates individualized dosing schemes for direct vs. indirect phenotyping in accordance with yet another embodiment of the present invention.

#### DETAILED DESCRIPTION OF THE INVENTION

20 The present invention refers to all drug metabolizing enzymes but will be exemplified in accordance with the metabolic pathways of the following enzymes: NAT1, NAT2, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, the metabolites of which are illustrated in FIGS 1-9. In particular, the present invention provides the ability to identify multiple phenotypic determinants of these enzymatic pathways and corresponding uses thereof. These enzymes are involved in the metabolism of large numbers of drugs, and as a result have important implications in the outcome of individual drug treatment regimes, clinical trial studies and the development and design of new drugs. These enzymes and their corresponding phenotypic determinants as described herein are provided as a representative example of determinants for the purposes of exemplifying the multi-determinant

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metabolic phenotyping of the present invention. However, the present invention is not limited thereto.

Table 1 lists a wide array of enzymes and corresponding medications that are known to be metabolized by the enzymes exemplified in accordance with the multi-determinant phenotyping of the present invention. These include drugs used for a variety of diseases, including asthma (theophylline), malaria (dapsone), breast cancer (tamoxifen), cardiovascular disease (procainimide), organ transplant (cyclosporine), common medications such as painkillers (acetaminophen, codeine), general anesthetics (lidocaine), and anxiolitics (valium). The wide array of medications that can be screened according to the multi-determinant metabolic phenotyping of the present invention is indicative of the impact that rapid phenotype screening can have on the success and safety of individual drug treatments in the future.

Table 1

Enzyme Specific Drug Metabolism

Enzyme	Drug
<b>NAT1</b>	p-aminobenzoic acid, p-aminosalicylic acid, dapsone
<b>NAT2</b>	Hydralzine, dapsone, caffeine, isoniazid, Amonafide
<b>CYP1A2</b>	Caffeine, theophylline, imipramine, propranolol, clozapine, 17 $\beta$ -estradiol (sex hormone), uroporphyrinogen, lidocaine, propafenone, tamoxifen (antiestrogen), Aminophylline, Amitriptyline, Betaxolol, Caffeine, Clomipramine, Clozapine, Chlorpromazine, 17 $\beta$ -estradiol (sex hormone), Fluvoxamine, Haloperidol, Imipramine, Lidocaine, Metoclopramide, Olanzapine, Ondansetron, Propafenone, Propranolol, Tacrine, Tamoxifen (antiestrogen), Theophylline, Thioridazine, Trifluoperazine, Uroporphyrinogen, Verapamil, ®-Warfarin
<b>CYP2A6</b>	Coumarin, nicotine, AZT
<b>CYP2C9</b>	Amitriptyline, Cerivastatin, Diclofenac, Fluoxetine, Fluvastatin, Ibuprofen, Losartan, Naproxen, Phenytoin, Piroxicam, Tamoxifen, D9-THC, Tolbutamide, Torsemide, (S)-Warfarin
<b>CYP2C19</b>	Phenyntoin, citalopram, diazepam, clomipramine, hexobarbitol, imipramine, dextromethorphan, propanolol, lansoprazole, omeprazole, pantoprazole, progesterone, sertraline, aminopyrine, proguanil, moclobemide
<b>CYP2D6</b>	<p><b>Psychotropic drugs:</b> amiflamine, amitriptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, trifluoperidol, zuclopenthixol.</p> <p><b>Cardiovascular agents:</b> bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylajmaline, propafenone, propranolol, sparteine, timolol, verapamil.</p> <p><b>Miscellaneous agents:</b> chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, phenformin.</p>
<b>CYP2E1</b>	Ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, p-nitrophenol
<b>CYP3A4</b>	Alprazolam, Amiodarone, Amitriptyline, Astemizole, Benzodiazepines, Budesonide, Bupropion, Buspirone, Caffeine, Carbamazepine, Cerivastatin, Cisapride, Clarithromycin, Clomipramine, Clonazepam, Codeine, Cyclosporine, Dexamethasone, Dextromethorphan, DHEA, Diazepam, Dihydropyridines, Diltiazem, Disopyramide, Donepezil, Doxorubicin, Doxycycline, Erythromycin, Estradiol, Ethinylestradiol, Etoposide, Felodipine, Fluoxetine, Imipramine, Lansoprazole, Lidocaine, Loratadine, Lovastatin, Midazolam, Nefazodone,

Nicardipine, Nifedipine, Nisoldipine, Norethindrone, Omeprazole, Ondansetron, Orphenadrine, Paclitaxel, Paroxetine, Progesterone, Propafenone, Quetiapine, Sildenafil, Simvastatin, Tacrolimus, Tamoxifen, Terfenadine, Testosterone, Theophylline, Trazodone, Triazolam, Venlafaxine, Verapamil, Vinblastine, ®-Warfarin, Zolpidem

Calcium Channel Blockers: Nifedipine, Diltiazem, Verapamil.

Further, many of these metabolic enzymes are responsible for the metabolism of carcinogenic compounds. Therefore, alterations in the activity of these enzymes alter the biological activity of many carcinogens. Table 2 lists the xenobiotics that are metabolized by the enzymes exemplified in accordance with the present invention.



**Table 2****Enzymes and the carcinogens they metabolize**

<b>Enzyme</b>	<b>Carcinogen</b>
<b>NAT1</b>	diaminobenzidine, N-hydroxy-4-aminobiphenyl; heterocyclic aromatic amines (MeIQx and PhIP)
<b>NAT2</b>	4-aminobiphenyl, diaminobenzidine, heterocyclic aromatic amines (MeIQx, PhIP)
<b>CYP1A2</b>	4-aminobiphenyl, heterocyclic amines (MeIQx, PhIP), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK, tobacco smoke product)
<b>CYP2A6</b>	Aflatoxin B1, N-nitrosodiethylamine, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK, tobacco smoke product),
<b>CYP2C9</b>	n-nitrosodimethylamine, benzo[a]pyrene, dibenzo[a,h]anthracene
<b>CYP2C19</b>	Does not appear to be a significant component in the metabolism of any carcinogenic compounds.
<b>CYP2D6</b>	Is involved in the metabolism of many carcinogens, however as yet is not reported as the major metabolizer for any.
<b>CYP2E1</b>	nitrosodimethylamine, nitrosopyrrolidone, benzene, carbon tetrachloride, 3-hydroxypyridine (tobacco smoke product)
<b>CYP3A4</b>	N'-nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), 5-Methylchrysene, 4,4'-methylene-bis(2-chloroaniline) (tobacco smoke products)

5        The factors influencing cancer development are multifactorial and it is difficult to associate a cancer with only one cause. However, current research has linked different metabolic phenotypes with increased risk of certain cancers.

10       Table 3 lists the xenobiotic metabolizing enzymes exemplified in accordance with the present invention having a phenotypic correlation to carcinogenesis. In

particular, Table 3 exemplifies specific enzymes and corresponding metabolic phenotypes that have been linked to an increased susceptibility for certain types of cancer.

5

**Table 3****Xenobiotic metabolizing enzymes associated with carcinogenesis**

Enzyme	Genotype Phenotype	/	Cancer	Comments
NAT1	NAT 10		Colorectal Bladder	OR = 1,9; 95% CI = 1.2-3.2 Metabolize benzidine
CYP1A2	Fast + NAT2	Fast	Colorectal	35% cases vs. 16% controls
CYP2A6	Slow		Tobacco related	Individuals carrying the CYP2A6 null alleles may have a decreased risk of developing tobacco-related cancers
CYP2D6	Fast + NAT2	Slow	Hepatocellular	OR = 2.6; 95% CI = 1.6-4.
CYP2E1	c2		Gastric	OR = 23.6-25.7

**CYP3A4**

The CYP 3A family constitutes approximately 25% of the total CYP 450 enzymes in the human liver.

10

**Polymorphism**

A large degree of inter-individual variability in the expression of the CYP3A4 isoenzymes has been shown in the human liver (>20 fold). However, the activity of CYP3A4 metabolism is distributed unimodally and as a result, there are currently no categorical classifications for distinct subsets of this population. Further, there is currently no evidence of a common allelic variant in the

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coding region of the gene. Recently, a rare allelic variant was identified in exon 7 (CYP3A4\*2). Limited data suggested that this mutation may result in altered substrate dependent kinetics compared with the wt CYP3A gene. It has been considered that the large inter-individual variability in the activity of CYP3A may reflect differences in transcriptional regulation. Another allelic variant in the 5'-flanking region of CYP3A has been identified (CYP3A4\*1B) that involves an A→G transition at position -290 from the transcriptional initiation site. It has been speculated that this nucleotide substitution may be associated with a reduced level of CYP3A activity. Ongoing studies are investigating the existence of a common allelic variant linked to CYP3A4 activity.

CYP3A4 metabolizes several drugs and dietary constituents including delavirdine, indinavir, ritonavir, saquinavir, amprenavir, zidovudine (AZT), nelfinavir mesylate, efavirenz, nevirapine, imiquimod, resiquimod, donepezil, lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin, rosuvastatin, benzafibrate, clofibrate, fenofibrate, gemfibrozil, niacin, benzodiazepines, erythromycin, dextromethorphan dihydropyridines, cyclosporine, lidocaine, midazolam, nifedipine, and terfenadine.

In addition, CYP3A4 activates environmental pro-carcinogens especially N'-nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK),

5-Methylchrysene, and 4,4'-methylene-bis(2-chloroaniline)  
(tobacco smoke products).

#### Induction and Inhibition

CYP3A4 is induced by a number of drugs including  
5 dexamethasone, phenobarbital, primidone and the antibiotic  
rifampicin. Conversely, CYP3A4 is inhibited by  
erythromycin, grapefruit juice, indinavir, ketoconazole,  
miconazole, quinine, and saquinavir.

#### Inter Ethnic Differences

10 Several studies have suggested that the activity of  
CYP3A4 varies between populations. Plasma levels of a  
CYP3A4 substrate drug after oral administration were  
reported to be twofold to threefold higher in Japanese,  
Mexican, Southeast Asian and Nigerian Populations compared  
15 with white persons residing in various countries. In  
addition, the CYP3A4\*1B allele has been reported to be  
more frequent in African-American populations as compared  
to European Americans or Chinese populations (66.7% vs.  
4.2% vs. 0%, respectively). The rare CYP3A4\*2 allele was  
20 found in 2.7% of a white population and was absent in the  
black and Chinese subjects. It is reasonable that, in  
drug metabolism studies, each ethnic group can be studied  
separately for evidence of polymorphism and its antimode  
should not be extrapolated from one ethnic population to  
25 another.

Due to the variability in CYP3A4 activity within the  
population it would be advantageous to be provided with a  
system and method for quickly and easily determining an

individual's CYP3A4 metabolic phenotype prior to administering a CYP3A4-dependant treatment thereto. In particular, such a system and method are believed to have enormous benefit in the individualization of therapy, and in particular with respect to the individualization of therapy with many hyperlipidia agents, including HMG-CoA reductase inhibitors (statins), fibrates, bile acid sequestrants and nicotinic acid (niacin).

#### Cyclosporine

10 An example of the need for phenotyping in drug dosing is the case of cyclosporine in the treatment of organ transplant individuals. Cyclosporine is an immunosuppressant agent (drug) administered post transplant to protect the new organ from being rejected. 15 Plasma levels of this drug are critical as high levels lead to renal toxicity but low levels can lead to organ rejection. Cyclosporine is metabolized via the CYP3A4 system. Several studies have indicated the importance of monitoring CYP3A4 activity in maintaining an effective and 20 safe cyclosporine dose. For these reasons, the utility of a reliable phenotyping test for CYP3A4 is evident.

#### Direct Phenotypic Determinants of CYP3A4

Different probe substrates can be used to determine the CYP3A4 phenotype (dapson, testosterone, nifedipine, 25 midazolam, erythromycin, dextromethorphan, cortisol). In accordance with the present invention, suitable probe substrates include without limitation, midazolam, dextromethorphan, erythromycin, dapson, testosterone, nifedipine and cortisol.

Of these midazolam is the preferred probe substrate. The structures of midazolam and its hydroxylated metabolite, 1'-hydroxymidazolam are illustrated in Fig. 1. In accordance with the present invention, the molar ratio  
5 of midazolam and its metabolite is used to determine the CYP3A4 phenotype of the individual as follows:

$$\frac{1'\text{-hydroxymidazolam}}{\text{midazolam}}$$

An individual's ratio will be considered as indicative  
10 of CYP3A4 enzyme activity with a lower ratio indicating poorer metabolism and a higher ratio indicating more extensive metabolism. The activity of CYP3A4 metabolism is distributed unimodally and hence no antimode is present. The levels of CYP3A4 activity as determined by direct  
15 phenotyping will be used.

#### Indirect Phenotypic Determinants of CYP3A4 (Genotyping)

To date only two mutant alleles have been identified for the CYP3A4 gene (CYP3A4\*1B and CYP3A4\*2). Studies have been unable to correlate these mutations with the  
20 large inter-individual variation in CYP3A4 activity. Despite confirmation in this regard to date, the use of indirect phenotyping is contemplated in accordance with the present invention. Ongoing studies continue to investigate this aspect of the present invention.

#### 25 NAT1

The NAT1 enzyme catalyzes the N-acetylation of many compounds. It is expressed in the liver as well as in mononuclear leucocytes.

### Polymorphism

The NAT1 gene was for a long time classified as monomorphic. However, it is now suggested that NAT1, like the other N-acetyltransferase gene (NAT2), is polymorphic.

5 Studies have demonstrated the presence of one wild type allele (NAT1\*4) and six mutant alleles (NAT1\*3, NAT1\*5, NAT1\*10, NAT1\*11, NAT1\*14 and NAT1\*17). NAT1 has two phenotypes: slow and rapid acetylators (e.g. NAT1\*4 vs. NAT1\*10 genotypes respectively).

10 NAT1 metabolizes several drugs and dietary constituents including p-aminobenzoic acid, p-aminosalicylic acid, and dapsone.

In addition, NAT1 activates environmental pro-carcinogens, especially diaminobenzidine, N-hydroxy-4-aminobiphenyl, and heterocyclic aromatic amines (MeIQx and PhIP). In one study it has been shown that individuals who have the NAT1\*10 allele, and hence are rapid N-acetylators, are at a greater risk for colorectal cancer (OR=1.9; 95% CI=1.2-3.2), while in another study they have  
15 an increased risk for bladder cancer (metabolize benzidine).  
20

### Inter Ethnic Differences

The activity of NAT1 varies broadly in a given population. Slow, and rapid NAT1 phenotypes have been distinguished. The NAT1\*10 genotype that is associated  
25 with rapid metabolic phenotype was monitored in three different ethnic populations, Indian, Malaysian and Chinese. The frequency of NAT1\*10 allele was 17%, 39% and 30%, respectively. The NAT1\*4 genotype, associated with

slow metabolizers, had a frequency in the same populations of 50%, 30% and 35%, respectively. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

#### Dapsone

A classical example of the need for phenotyping in drug dosing is the case of dapsone. Dapsone is used in the treatment of malaria and is being investigated for the treatment of *Pneumocystis carinii* pneumonia in AIDS individuals. Adverse effects include rash, anemia, methemoglobinemia, agranulocytosis, and hepatic dysfunction. Dapsone is cleared from the body via the NAT1 metabolizing system. A study has shown a correlation between slow acetylation and increased adverse reactions to dapsone (46% vs. 17% for slow and fast acetylators, respectively). For these reasons, the utility of a reliable phenotyping test is evident.

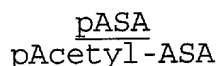
#### Phenotypic Determinants of NAT1

Different probe substrates can be used to determine the NAT1 phenotype, such as (p-aminosalicylic acid (pASA), and p-aminobenzoic acid (pABA)). In accordance with the present invention suitable probe substrates include, without limitation, p-aminosalicylic acid, and p-aminobenzoic acid.



Of these pASA is the preferred probe substrate. The structure of pASA and its acetylated metabolite p-acetylaminosalicylic acid are illustrated in Fig. 2.

In accordance with the present invention, the molar ratio of pASA and its acetylated metabolite is used to determine the NAT1 phenotype of the individual as follows:



#### Indirect Phenotypic Determinants of NAT1 (Genotyping)

10 The NAT1 alleles NAT1\*4 (wt) and the mutant NAT1\*14 can be determined either by PCR-RFLP or allele specific PCR (Hickman, D. et al. (1998); Gut 42:402-409). The PCR-RFLP methodology requires the amplification of the fragment of gene containing the A560G mutation. This is performed with the following primers:

5'-TCCTAGAAGACAGCAACGACC-3'  
5'-GTGAAGCCCACCAAACAG-3'

This PCR amplification produces a 175 bp fragment that is incubated with the *BsaI* restriction enzyme. The Nat1\*4 allele is cleaved and produces a 155 bp fragment and a 20 bp fragment, while the mutant NAT1\*14 is uncleaved.

The NAT1\*14 allele is confirmed using an allele specific PCR, with the following primers:

25 5'-TCCTAGAAGACAGCAACGACC-3'  
5'-GGCCATCTTTAAATACATTTT-3'

#### CYP1A2

CYP1A2 constitutes 15% of the total CYP 450 enzymes in the human liver.

### Polymorphism

CYP1A2 may be polymorphic although it remains to be established firmly. To date no mutant alleles have been identified. Three metabolic phenotypes can be distinguished: rapid, intermediate and slow metabolizers. CYP1A2 metabolizes several drugs and dietary constituents including resiquimod, imiquimod, tacrine, acetaminophen, anti pyrine, 17  $\beta$ -estradiol, caffeine, cloipramine, clozapine, flutamide (antiandrogenic), imipramine, paracetamol, phenacetin, tacrine and theophylline.

In addition, CYP1A2 activates environmental pro-carcinogens, especially heterocyclic amines and aromatic amines. In one study it has been shown that individuals who are fast N-acetylators and have high CYP1A2 activity are at a greater risk for colorectal cancer (35% of cases vs. 16% of controls, OR=2.79 (P=0.00-2)).

### Induction and Inhibition

CYP1A2 is induced by a number of drugs and environmental factors such as omeprazole, lansoprasole, polyaromatic hydrocarbons and cigarette smoke. CYP1A2 is inhibited by oral contraceptives, ketoconazole,  $\alpha$ -naphthoflavone, fluvoxamine (serotonine uptake inhibitor), and furafylline.

### Inter Ethnic Differences

The activity of CYP1A2 varies broadly (60 to 70 fold) in a given population. Slow, intermediate and rapid CYP1A2 phenotypes have been distinguished. The proportion of these three CYP1A2 phenotypes varied between ethnic

groups and countries: % of intermediates: 50, 70, 60, >95, 60, 20 in U.S.A., African-American, China, Japan, Italy and Australia, respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

### Theophylline

A classical example of the need for phenotyping in drug dosing is the case of theophylline. Theophylline is used in the treatment of asthma. However, theophylline toxicity continues to be a common clinical problem, and involves life-threatening cardiovascular and neurological toxicity. Theophylline is cleared from the body via the CYP1A2 metabolizing system. Inhibition of CYP1A2 by quinolone antibiotic agents or serotonin reuptake inhibitors may result in theophylline toxicity. For these reasons, the utility of a reliable phenotyping test for CYP1A2 is evident.

### Direct Phenotypic Determinants of CYP1A2

Different probe substrates can be used to determine the CYP1A2 phenotype (caffeine, theophylline). In accordance with the present invention suitable probe substrates include without limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe substrate. Caffeine is widely consumed and relatively safe. The structure of caffeine and its metabolites 1,7-

dimethylxanthine (1,7 DMX) and 1,7-dimethyluric acid (1,7 DMU) are illustrated in Fig. 3.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the CYP1A2 phenotype of the individual as follows:

$$\frac{1,7\text{-dimethylxanthine (1,7 DMX)} + 1,7\text{-dimethyluric acid (1,7 DMU)}}{\text{caffeine}}$$

Molar ratios of 4 and 12 separate slow, intermediate and fast CYP1A2 metabolizers, respectively (Butler et al. (1992) Pharmacogenetics 2:116-117).

#### Indirect Phenotypic Determinants of CYP1A2 (Genotyping)

To date no mutant alleles have been identified for the CYP1A2 gene. Therefore, indirect phenotyping is not currently possible for CYP1A2.

#### CYP2A6

CYP2A6 constitutes 4% of the total CYP 450 enzymes in the human liver. CYP2A6 is estimated as participating in 2.5% of drug metabolism.

#### Polymorphism

CYP2A6 is functionally polymorphic with two mutant alleles, CYP2A6\*2 and CYP2A6\*3, resulting in an inactive enzyme or the absence of the enzyme, respectively. Two metabolic phenotypes can be distinguished: poor and extensive metabolizers. CYP2A6 metabolizes several drugs including neuroleptic drugs and volatile anesthetics as well as the natural compounds, coumarin, nicotine and aflatoxin B1.

In addition, CYP2A6 activates several components of tobacco smoke (e.g. NNK), as well as 6-aminochrysene. The role of activation of tobacco smoke and the metabolism of nicotine have suggested a role for CYP2A6 in the development of smoking related cancers.

#### Induction and Inhibition

CYP2A6 is induced by barbiturates, antiepileptic drugs and corticosteroids.

#### Inter Ethnic Differences

CYP2A6 demonstrates marked inter-individual variability and has demonstrated ethnic related differences. The proportion of the two phenotypes varied between ethnic groups and countries: % of wt genotype (extensive metabolizers): 85, 76, 52, 83, 97.5 in Finnish, English, Japanese, Taiwanese and African-American populations, respectively. It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

#### Nicotine

An example of the need for phenotyping in drug dosing is in the delivery of nicotine, for a smoking cessation program. CYP2A6 is the primary means of nicotine metabolism. Extensive CYP2A6 metabolizers will eliminate nicotine at a much higher rate. Identification of individuals with an increased CYP2A6 activity and hence increased nicotine metabolism may identify those

individuals that will require higher doses of nicotine at the onset of their attempt to quit smoking with the assistance of a nicotine delivery system. Alternatively, these individuals may benefit from non-nicotine delivery systems for assisting in quitting smoking.

#### Direct Phenotypic Determinants of CYP2A6

A probe substrate can be used to determine the CYP2A6 phenotype (coumarin). In accordance with the present invention suitable probe substrates include, without limitation, coumarin. The structure of coumarin and its metabolite 7-hydroxycoumarin are illustrated in Fig. 4.

In accordance with the present invention, the molar ratio of coumarin and its metabolite, 7-hydroxycoumarin is used to determine the CYP2A6 phenotype of the individual as follows:

$$\frac{\text{7-hydroxycoumarin}}{\text{coumarin}}$$

#### Indirect Phenotypic Determinants of CYP2A6 (genotyping)

Currently three alleles have been identified for the CYP2A6 gene, the wild type allele (CYP2A6\*1) and two mutant alleles (CYP2A6\*2, and CYP2A6\*3). The wt allele codes for a fully functional enzyme. The CYP2A6\*2 mutant allele codes for an inactive enzyme and the CYP2A6\*3 allele does not produce any enzyme.

Determination of an individual genotype can be performed by a combined LA-PCR and PCR-RFLP procedure. In this procedure, specific oligonucleotide primers were used to amplify the CYP2A6/7 gene. The amplified CYP2A6/7 gene

is then used as the PCR template to amplify exons 3 and 4 using specific oligonucleotide primers to amplify a 544 bp fragment. This fragment is then digested with the *FspI* restriction enzyme and a 489 bp fragment re-isolated.

- 5 This 489 bp fragment is then incubated with both *DdeI* and *XcmI*. The digestion patterns were determined by electrophoresis. The wildtype allele produces 330, 87 and 72 bp fragments, the CYP2A6\*2 allele yields 189, 141, 87 and 72 bp fragments and the CYP2A6\*3 allele yields 270, 87, 72, 60 bp fragments (Nakajima et al. (2000) Clin Pharmacol & Ther. 67(1):57-69).

#### Primers

##### CYP2A6/7 LA-PCR

- 5' - CCTCCCTTGCTGGCTGTGTCCCAAGCTAGGC - 3'  
15 5' - CGCCCCTTCCTTTCGCCATCCTGCCCCCAG - 3'

##### Exon 3 PCR

5' - GCGTGGTATTCAGCAACGGG - 3'  
5' - TGCCCCGTGGAGGTTGACG - 3'

#### CYP2C19

- 20 CYP2C19 accounts for about 2% of oxidative drug metabolism. CYP2C19 has been postulated as participating in ~8% of drug metabolism.

#### Polymorphism

- 25 Individuals are genetically polymorphic with respect to CYP2C19 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. Two genetic polymorphisms have been identified (CYP2C19\*2 and CYP2C19\*3) that together explain all of the Oriental poor metabolizers and about 83% of Caucasian poor metabolizers.

Both of these mutations introduce stop codons resulting in a truncated and non-functional enzyme.

CYP2C19 metabolizes a variety of compounds including the tricyclic antidepressants amitriptyline, imipramine and clomipramine, the sedatives diazepam and hexobarbital, the gastric proton pump inhibitors, omeprazole, pantoprazole, and lansoprazole, as well as the antiviral nelfinavir mesylate, the antimalarial drug proguanil and the  $\beta$ -blocker propranolol.

#### 10 Induction and Inhibition

CYP2C19 is inhibited by fluconazole, fluvoxamine, fluoxetine, sertraline, and ritonavir. It is induced by rifampin.

#### Inter Ethnic Differences

15 The occurrence of the poor metabolizer phenotype for CYP2C19 shows a large inter ethnic variability. Poor metabolizers make up less than 4% of the European and white American populations. While the Korean population has a poor metabolizer frequency of 12.6%, the Chinese 17.4% and the Japanese 22.5%. In addition, the CYP2C19 mutant alleles demonstrate interethnic variability with CYP2C19\*2 frequency ranging from 28.9% in the Chinese population to only 13% in European-American population. The CYP2C19\*3 allele is absent from the European-American or African-American populations, while occurring at a frequency of 11.7% in both the Korean and Japanese populations.



It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

5 Omeprazole

As an example, the benefit of CYP2C19 metabolic phenotyping in drug dosing is evident in the case of omeprazole. Omeprazole is a drug used in the treatment of *Helicobacter pylori* (*H pylori*) infections in conjunction with amoxicillin, and is cleared from the body via a CYP2C19 metabolic pathway. Studies have observed higher eradication rates of in CYP2C19 poor metabolizers. Therefore, extensive metabolizers may require higher doses of omeprazole to achieve the same level of *H pylori* eradication observed in poor metabolizers. For these reasons, the utility of a reliable phenotyping test for CYP2C19 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

Direct Phenotypic Determinants of CYP2C19

In accordance with an embodiment of the present invention, the ratio of S-(+)mephenytoin and R-(-)mephenytoin in an urine sample may be used to provide a determination of an individual's CYP2C19 phenotype. These metabolites are used as quantitative markers in the determination of a CYP2C19 phenotype on the basis of the use of the preferred probe substrate mephenytoin.

However, it is fully contemplated that the present invention is not limited in any respect thereto.

The structure of R-(-) and S-(+) mephenytoin and 4-hydroxymephenytoin are illustrated in Fig. 5.

- 5 The chiral ratio of S-(+)mephenytoin and R-(-)mephenytoin metabolites, used to determine the CYP2C19 phenotype of the individual, is as follows:

$$\frac{\text{S-(+)Mephenytoin}}{\text{R-(-)Mephenytoin}}$$

- 10 Chiral ratios of close to unity ( $>0.8$ ) are indicative of fast CYP2C19 metabolizers.

#### Indirect Phenotypic Determinants of CYP2C19 (Genotyping)

- As mentioned previously the CYP2C19 has two predominant variant alleles, which account for all
- 15 Japanese poor metabolizers and 83% of Caucasian poor metabolizers. Studies have demonstrated an excellent correlation between a homozygous presence of mutant alleles and poor metabolizer status. An example of a procedure for genotyping CYP2C19 involves a series of
- 20 polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2C19\*1 allele (Furuta *et al.* (1999) Clin Pharmacol Thera 65(5):552-561; Tanigawara *et al.* (1999)
- 25 Clin Pharmacol Thera 66(5):528-5534). PCR amplification of exon 5 or exon 4 for CYP2C19\*2 and CYP2C19\*3 respectively are performed using the following primers:

## CYP2C19\*2 Exon 5 Primers

5'-AATTACAACCAGAGCTTGGC-3'

5'-TATCACTTTCCATAAAAGCAAG-3'

## CYP2C19\*3 Exon 4 Primers

5 5'-AACATCAGGATTGTAAGCAC-3'

5'-TCAGGGCTTGGTCAATATAG-3'

The presence of the G681A mutation in CYP2C19\*2 is then detected by digestion with the *Sma*I restriction enzyme. The wild type allele will produce a 120 and a 49 bp fragment, while the CYP2C19\*2 allele will remain uncleaved. The CYP2C19\*3 allele is detected by incubating the exon 4 PCR product with *Bam*HI. The wild type allele will produce a 233 bp and a 96 bp fragment while the CYP2C19\*3 allele will remain uncleaved.

Extensive metabolizing phenotype is assigned to those individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2C19 alleles.

## 20 CYP 2C9

The CYP2C9 family of metabolic enzymes accounts for approximately 8% of the metabolic enzymes in the liver. CYP 2C9 has been postulated as participating in approximately 15% of drug metabolism.

## 25 Polymorphism

Individuals are genetically polymorphic with respect to CYP 2C9 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. Three genetic polymorphisms have been definitively identified, one wild type (CYP2C9\*1) and two mutant (CYP2C9\*2 and

CYP2C9\*3). The CYP2C9\*2 allele was found to result in 5-10 fold increase in expression of mRNA and have 3-fold higher enzyme activity for metabolism of phenytoin and tolbutamide. Conversely, this genotype appears to have a lower level of activity for the metabolism of S-warfarin. The CYP2C9\*3 allele appears to demonstrate decreased metabolic activity against all three of these substrates.

CYP2C9 metabolizes a variety of compounds including S-warfarin, phenytoin, tolbutamide, tienilic acid, and a number of nonsteroidal antiinflammatory drugs such as diclofenac, piroxicam, tenoxicam, ibuprofen, and acetylsalicylic acid.

#### Induction and Inhibition

CYP2C9 is inhibited by fluconazole, metronidazole, miconazole, ketoconazole, itaconazole, ritonavir, clopidrogel, amiodarone, fluvoxamine, sulfamthoxazole, fluvastatin and fluoxetine. It is induced by rifampin and rifabutin.

#### Inter Ethnic Differences

The CYP2C9 genotypes demonstrate marked inter ethnic variability. The CYP2C9\*2 is absent from Chinese and Taiwanese populations and present in only 1% of African American populations, but accounts for 19.2% of the British population and 8% of Caucasians. CYP2C9\*3 is rarer and is present in 6% of Caucasian, 2% of Chinese, 2.6% of Taiwanese and 0.5% of African-American populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence

of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

#### S-warfarin

As an example, the benefit of CYP2C9 metabolic phenotyping in drug dosing is evident in the case of S-warfarin. S-warfarin is an anticoagulant drug. Studies have demonstrated that the presence of either CYP2C\*2 or CYP2C9\*3 haplotypes results in a decrease in the dose necessary to acquire target anticoagulation intensity. In addition, these individuals also suffered from an increased incidence of bleeding complications. Therefore, the CYP2C9 gene variants modulate the anticoagulant effect of the dose of warfarin prescribed. For these reasons, the utility of a reliable test for CYP2C9 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective treatment regimes for individuals on an individual basis.

#### Direct Phenotypic Determinants of CYP2C9

In accordance with an embodiment of the present invention, the ratio of (S)-ibuprofen and its carboxylated metabolite, (S)-2-carboxyibuprofen in a urine sample may be used to provide a determination of an individual's CYP2C9 phenotype. These metabolites are used as quantitative markers in the determination of a CYP2C9 phenotype on the basis of the use of the preferred probe substrate (S)-ibuprofen. The structures of (S)-ibuprofen and its metabolite (S)-2-carboxyibuprofen are illustrated in Fig. 6. However, it is fully contemplated that the

present invention is not limited in any respect thereto.

In fact, due to the nature of the substrate specific alterations caused by the individual CYP2C9 mutations, multiple probe substrates may be necessary for a

5 completely informative phenotypic determination of CYP2C9.

The molar ratio of (S)-ibuprofen and its (S)-2-carboxyibuprofen metabolite, used to determine the CYP2C9 phenotype of the individual, is as follows:

(S)-ibuprofen

10 (S)-2-carboxyibuprofen

Indirect Phenotypic Determinants of CYP2C9 (Genotyping)

As mentioned previously the CYP2C9 has two predominant variant alleles, CYP2C9\*2 and CYP2C9\*3. An example of a procedure for genotyping CYP2C9 involves a series of  
15 polymerase chain reaction - restriction fragment length polymorphism reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2C9\*1 allele (Taube et al. (2000) Blood 96(5):1816-1819). PCR amplification of exon 3 for  
20 CYP2C9\*2 is performed using the following primers:

CYP2C9\*2 Exon 3 Primers

5'-CAATGGAAAGAAATGGAAGGAGGT-3'

5'-AGAAAGTAATACTCAGACCAATCG-3'

25 A forced mismatch was included in the penultimate base of the forward primer to create a restriction site for the AvaII digestion. The PCR product from this amplification is 251 bp in length. After AvaII digestion the CYP2C9\*1

(wt) allele produces 170 and 60 bp fragments. The CYP2C\*2 allele produces a 229 bp fragment.

The CYP2C9\*3 allele does not naturally destroy or produce a restriction site. Therefore, a restriction site was introduced into the forward primer such that the adenosine at position 1061 (A1061) in combination with the mismatch creates a restriction site for the *NsiI* restriction enzyme. Therefore the PCR amplified fragment of the CYP2C9\*1 (wt) allele would have a restriction site at A1061. Conversely, the mutation of A1061C in CYP2C9\*3 removes this restriction site. The forward primer also includes a natural *AvaII* restriction sequence. The reverse primer also has a forced mismatch at 1186 to provide a restriction site for the *NsiI* restriction enzyme (PCR amplified fragments from both the CYP2C9\*1 and CYP2C9\*3 alleles will have this restriction site). The PCR product for this set of primers prior to restriction enzyme digest is 160 bp in length. Following restriction digest with *NsiI* and *AvaII*, the CYP2C9\*1 allele produces a 130 bp fragment and the CYP2C9\*3 allele produces a 140 bp fragment.

#### CYP2C9\*3 Primers

5'-TGCACGAGGTCCAGAGATGC-3'

25 5'-AGCTTCAGGGTTTACGTATCATAGTAA-3'

Due to the substrate specific alterations in enzyme activity resulting from the two allelic variants, the phenotypic determination will be correlated on an individual substrate basis.

## CYP2D6

CYP2D6 constitutes 1-3% of the total CYP 450 enzymes in the human liver. CYP2D6 has been postulated as participating in ~20% of drug metabolism.

## 5 Polymorphism

CYP2D6 was the first P450 enzyme to demonstrate polymorphic expression in humans. Three metabolic phenotypes can be distinguished: poor, (PM), extensive (EM) and ultraextensive (UEM) phenotypes. The CYP2D6 gene is extensively polymorphic. For example, a 1997 study documented 48 mutations and 53 alleles of the CYP2D6 gene in a screen of 672 unrelated individuals. Examples of alleles with normal (extensive), wild-type function are CYP2D6\*1, CYP2D6\*2A, and CYP2D6\*2B; alleles resulting in an absence of function are CYP2D6\*3, CYP2D6\*4A, CYP2D6\*4B, CYP2D6\*5, CYP2D6\*6A, CYP2D6\*6B, CYP2D6\*7, CYP2D6\*8, CYP2D6\*11 and CYP2D6\*12; and alleles resulting in a reduced function are CYP2D6\*9, CYP2D6\*10A, and CYP2D6\*10B. The ultraextensive phenotype appears to arise from the presence of multiple copies of the CYP2D6 gene (for example, one individual was identified with 13 copies of the gene).

CYP2D6 metabolizes a large variety of drugs and dietary constituents including, but not limited to the following:



## Antiviral agents:

Efavirenz, nevirapine, ritonavir, saquinovir, nelfinavir mesylate, and indinavir

## Psychotropic drugs:

- 5 amiflamine, amitriptyline, clomipramine, clozapine, desipramine, haloperidol, imipramine, maprotiline, methoxyphenamine, minaprine, nortriptyline, paroxetine, perphenazine, remoxipride, thioridazine, tomoxetine, trifluoperidol, zuclopenthixol, risperidone, fluoxetine.

## 10 Cardiovascular agents:

aprimidine, bufuralol, debrisoquine, encainide, flecainide, guanoxan, indoramin, metoprolol, mexiletin, n-propylamaline, propafenone, propranolol, sparteine, timolol, verapamil.

## 15 Miscellaneous agents:

chlorpropamide, codeine, dextromethorphan, methamphetamine, perhexilene, phenformin.

- 20 In addition, CYP2D6 is involved in the metabolism of many carcinogens, however, as yet it is not reported as the major metabolizer for any. In one study it has been shown that individuals who are fast CYP2D6 metabolizers and slow N-acetylators are at a greater risk for hepatocellular cancer (OR=2.6; 95% CI=1.6-4).

## Induction and Inhibition

- 25 CYP2D6 is inhibited *in vitro* by quinidine and by viral protease inhibitors as well as by appetite suppressant drugs such as D- and L-fenfluramine.

### Inter Ethnic Differences

The activity of CYP2D6 varies broadly in a given population. Poor (PM), extensive (EM) and ultraextensive (UEM) phenotypes of CYP2D6 have been distinguished. The CYP2D6 gene is inherited as an autosomal recessive trait and separates 90 and 10% of the white European and North American population into extensive (EM) and poor (PM) metabolizer phenotypes, respectively. In another study the percentage of PM in different ethnic populations was observed, and white North Americans and Europeans were found to have 5-10% PM's, African-American, 1.8%, Native Thais, 1.2%, Chinese 1%, and Native Malay populations, 2.1%, while the PM phenotype appears to be completely absent in the Japanese population.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

### Dextromethorphan/Antidepressants

An example of the need for phenotyping in drug dosing is the case of dextromethorphan. Dextromethorphan is a nonopioid antitussive with psychotropic effects. However, dextromethorphan doses range from 0 to 6 mg/kg based on individual subject tolerance. Dextromethorphan is activated via the CYP2D6 metabolizing system. Dextromethorphan produced qualitatively and quantitatively different objective and subjective effects in poor vs. extensive metabolizers (mean performance  $\pm$  SE, 95 $\pm$ 0.5% for EMs vs. 86 $\pm$ 6% for PMs;  $p < 0.05$ ).

Another important class of drugs for CYP2D6 phenotyping is the tricyclic antidepressants. Both the PM and UEM phenotypes of CYP2D6 are at risk of adverse reactions. PM individuals given standard doses of these drugs will develop toxic plasma concentrations, potentially leading to unpleasant side effects including dry mouth, hypotension, sedation, tremor, or in some cases life-threatening cardiotoxicity. Conversely, administration of these drugs to UEM individuals may result in therapeutic failure because plasma concentrations of active drugs at standard doses are far too low. For, these reasons, the utility of a reliable phenotyping test for CYP2D6 is evident.

#### Phenotypic Determinants of CYP2D6

Different probe substrates can be used to determine the CYP2D6 phenotype (dextromethorphan, debrisoquine, bufuralol, antipyrine, theophylline and hexobarbital). In accordance with the present invention, suitable probe substrates include without limitation, dextromethorphan, debrisoquine, and bufuralol.

Of these dextromethorphan is the preferred probe substrate. The structure of dextromethorphan and its demethylated metabolite dextrorphan are illustrated in Fig. 7.

In accordance with the present invention, the molar ratio of dextromethorphan and its metabolite is used to determine the CYP2D6 phenotype of the individual as follows:

dextromethorphan  
dextrorphan

An antinode of 0.30 is used to differentiate between extensive and poor metabolizers whereby an antinode of  
5 less than 0.30 indicates an extensive metabolizer and greater than 0.30 indicates a poor metabolizer.

Indirect Phenotypic Determinants of CYP2D6 (Genotyping)

As mentioned previously the CYP2D6 gene is extensively  
polymorphic with one study identifying 48 mutations and 53  
10 alleles. An example of a procedure for genotyping CYP2D6 involves the amplification of the entire CYP2D6 coding region (5.1kb product) by XL-PCR using specific primers. This product is then used for a series of polymerase chain reaction - restriction fragment length polymorphism  
15 reactions designed to detect nucleotide point mutations, deletions and insertions compared with the functional CYP2D6\*1 allele (Garcia-Barceló *et al.* (2000) *Clinical Chemistry* 46(1):18-23). For example, to detect the C188T transition mutation the following primers can be used to  
20 first amplify the CYP2D6 gene and then the specific region of the mutation:

Full CYP2D6 gene

5'-CCAGAAGGCTTTGCAGGCTTCA-3'

5'-ACTGAGCCCTGGGAGGTAGGTA-3'

25 C188T Mutation

5'-CCATTTGGTAGTGAGGCAGGTAT-3'

5'-CACCATCCATGTTTGCTTCTGGT-3'

The presence of the C188T mutation is then detected by digestion with the *HphI* restriction enzyme.

In general, the most frequent mutations are examined and these correspond to the most frequent alleles and genotypes.

Extensive metabolizing phenotype is assigned to those  
5 individuals with at least one allele encoding a functional enzyme. The poor metabolizing phenotype is assigned to individuals lacking two or more functional CYP2D6 alleles.

#### CYP2E1

CYP2E1 constitutes approximately 5% of the total CYP  
10 450 enzymes in the human liver.

#### Polymorphism

The CYP2E1 gene has been demonstrated to be polymorphic in the human population. Studies have demonstrated the presence of 10 CYP2E1 alleles (one wt  
15 CYP2E1\*1, and 9 mutant, CYP2E1\*2, CYP2E1\*3, CYP2E1\*4, CYP2E1\*5A, CYP2E1\*5B, CYP2E1\*6, CYP2E1\*7A, CYP2E1\*7B, and CYP2E1\*7C). The exact relationship of these polymorphisms to CYP2E1 enzyme activity has not been clarified, however, some studies suggest that the mutant alleles CYP2E1\*5A and  
20 CYP2E1\*5B, result in increased transcription and increased enzyme activity.

CYP2E1 metabolizes several drugs and dietary constituents including isoflurane, halothane, methoxyflurane, enflurane, propofol, thiamylal,  
25 sevoflurane, ethanol, acetone, acetaminophen, nitrosamines, nitrosodimethylamine, and p-nitrophenol.

In addition, CYP2E1 activates environmental pro-carcinogens especially nitrosodimethylamine,

nitrosopyrrolidone, benzene, carbon tetrachloride, and 3-hydroxypyridine (tobacco smoke product). In one study it has been shown that individuals who have high CYP2E1 (CYP2E1\*5A or CYP2E1\*5B) activity are at a greater risk for gastric cancer (OR=23.6-25.7).

#### Induction and Inhibition

CYP2E1 is induced by a number of drugs and environmental factors such as cigarette smoke as well as by starvation, chronic alcohol consumption and in uncontrolled diabetes. CYP2E1 is inhibited by chlormethiazole, trans-1,2-dichloroethylene, disulferan (cimetidine) and by the isoflavonoids genistein and equol.

Induction or inhibition by environmental factors can severely alter an individual's capacity to metabolize certain drugs. Therefore, the present invention may find further application in the individualization of therapy whereby environmental factors are determined to effect an individual's metabolism specific to an enzyme and/or metabolic pathway of interest with respect to a given drug, such as CYP2E1, for example. Furthermore, as environmental factors vary on an individual basis and over time, the present invention may be employed to detect changes in an individual's metabolism specific to an enzyme and/or metabolic pathway of interest due to environmental factors at any given time, and provide valuable phenotype-specific information in the determination of a safe and efficacious individualized treatment regime. By employing the present invention on a routine basis, an individual's treatment regime may be

modified to account for environmental influences and maximize the effectiveness of treatment.

#### Inter Ethnic Differences

The proportion of CYP2E1 phenotypes varied between ethnic groups and countries: the frequency of the rare c2 (CYP2E1\*5A or CYP2E1\*5B) allele is about 4% in Caucasians and 20% in the Japanese and a study of a separate polymorphism described a rare C allele (CYP2E1\*5A or CYP2E1\*6) that has a frequency of about 10% in Caucasian and 25% in Japanese populations. In one study it was shown that Japanese males had much lower levels of CYP2E1 activity as compared to Caucasian males. Therefore, it is reasonable that, in drug metabolism studies, each ethnic group can be studied separately for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another.

#### Acetaminophen

An example of the need for phenotyping in drug dosing is the case of acetaminophen. Acetaminophen is a widely used painkiller. However, acetaminophen causes hepatotoxicity at low frequency. The hepatotoxicity is due to its transformation via CYP2E1, to a reactive metabolite (N-acetyl-p-benzoquinoneimine) which is capable of binding to nucleophiles. For these reasons, the utility of a reliable phenotyping test for CYP2E1 is evident.

### Direct Phenotypic Determinants of CYP2E1

In accordance with the present invention a suitable probe substrate is, without limitation, chlorzoxazone.

In accordance with the present invention, the molar  
5 ratio of chlorzoxazone and its metabolite is used to determine the CYP2E1 phenotype of the individual as follows:

$$\frac{\text{6-hydroxychlorzoxazone}}{\text{chlorzoxazone}}$$

10 The structures of chlorzoxazone and its metabolite 6-hydroxychlorzoxazone are illustrated in Fig. 8.

### Indirect Phenotypic Determinants of CYP2E1 (Genotyping)

As mentioned previously the CYP2E1 gene has multiple polymorphisms. An example of a procedure for genotyping  
15 CYP2E1 for the most common mutations, those termed the *Pst*/*Rsa*I and *Dra*I mutations (allows genotyping of CYP2E1\*5A, CYP2E1\*5B and CYP2E1\*6), involves the amplification of a fragment containing either the *Pst*I and *Rsa*I restriction sites or the *Dra*I restriction site using  
20 specific primers (Nedelcheva et al. (1996) Methods in Enzymology 272:218-225). The amplified product is then incubated with the appropriate restriction enzyme (*Pst*I or *Rsa*I/*Dra*I) and the digestion products separated electrophoretically. From an allele with wt sequence at  
25 the *Pst*I or *Rsa*I site, the 510 bp fragment produced by PCR is cleaved to a 360 bp and a 150 bp fragment. From the mutant allele the 510 bp fragment remains uncleaved. From an allele with the wt sequence at the *Dra*I mutation site, the 370 bp PCR amplified fragment is cleaved to a 240 bp



and 130 bp pair of fragments, while the mutant allele is uncleaved.

*Pst*I/*Rsa*I Primers

5'-CCCGTGAGCCAGTCGAGT-3'

5'-ATACAGACCCTCTTCCAC-3'

*Dra*I Primers

5'-AGTCGACATGTGATGGATCCA-3'

5'-GACAGGGTTTCA-TCATGTTGG-3'

The CYP2E1\*5A mutant allele contains both the *Rsa*I and the *Dra*I mutations, while the CYP2E1\*5B mutant allele contains the *Rsa*I mutation alone. The *Rsa*I mutation has been associated with an increased expression and increased enzyme activity. Therefore, an individual with two copies of either CYP2E1\*5 allele could be considered assigned an extensive metabolizing phenotype. Conversely, the CYP2E1\*2 mutation has been associated with decreased protein expression and decreased enzyme activity. Therefore, a person homozygous for the CYP2E1\*2 allele could be assigned a poor metabolizing phenotype.

NAT2

Polymorphism

Individuals are genetically polymorphic in their rate of N-acetylation of drugs via the N-acetyltransferase (NAT2) pathway (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). Two major metabolic phenotypes can be distinguished: fast and slow N-acetylators. Drugs that are subject to N-acetylation polymorphism include sulfonamides (sulfamethazine), antidepressants (phenelzine), antiarrhythmics (procainamide), and antihypertensives (hydrazine). Some adverse therapeutic consequences of the acetylator phenotype are peripheral

neuropathy and hepatitis. In an opposite manner, the N-acetylation of procainamide produces a therapeutically active metabolite with reduced toxicity. N-acetylation polymorphism has also been linked to the detoxification pathway of some environmental carcinogenic arylamines and there is a higher frequency of bladder cancers among chemical dye workers who are slow N-acetylators.

The NAT2 gene is polymorphic, there have been 9 mutations detected and 14 mutant alleles. Six mutant alleles are responsible for 99% of Caucasian slow acetylators (NAT2\*5A, NAT2\*5B, NAT2\*5C, NAT2\*6A, NAT2\*7B, and NAT2\*13). The NAT2\*4 allele is the wild-type allele.

#### Inter Ethnic Differences

The frequencies of PM (poor metabolizer) and EM (extensive metabolizers) (autosomal recessive trait) show considerable inter ethnic differences for the N-acetylation polymorphism. In Caucasians, the frequencies are approximately 60 and 40%, respectively, while in Orientals, they are 20 and 80%, respectively (Meyer, U.A. (1994) Proc. Natl. Acad. Sci. USA, 91:1983-1984). It is reasonable that, in drug metabolism studies, each ethnic group is studied separately for evidence of polymorphism and its antinode should not be extrapolated from one ethnic population to another.

#### 25 Direct Phenotyping - Phenotypic Determinants of NAT2

Different probe substrates can be used to determine the NAT2 phenotype. In accordance with the present invention a suitable probe substrate is, without

limitation caffeine. Caffeine is widely consumed and relatively safe. A phenotype may be generally determined from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X) present in urine samples of an individual collected after drinking coffee. The structures of these metabolites are illustrated in Fig. 9. The ratio of these metabolites provides a determination of an individual's N-acetylation (NAT2) phenotype.

$$\text{AAMU (or AFMU) / 1X}$$

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the acetylation phenotype of the individual as follows. Individuals with a ratio less than 1.80 are slow acetylators.

#### Indirect Phenotyping (Genotyping)

An example of NAT2 genotyping involves the amplification of a 547 bp fragment which includes the 5 of the 6 mutant alleles which are responsible for 99% of Caucasian slow acetylators. Analysis of these 5 alleles and the wt allele can be performed by examining 4 mutations (Smith CAD et al. J Med Genet (1997) 34:758-760).

The PCR amplification is performed with the following primers:

5' -GCTGGGTCTGGAAGCTCCTC-3'

5' -TTGGGTGATACATACACAAGGG-3'

The analysis of this fragment with 4 restriction digestion enzymes allows the detection of 6 alleles (NAT2\*4 (wt) and the mutants NAT2\*5A, NAT2\*5B, NAT2\*5C, NAT2\*6 and NAT2\*7). Each of the 6 alleles have distinct combinations of the mutations and as each mutation alters a specific restriction digestion enzyme site (*KpnI*, *DdeI*, *TaqI* or *BamHI*), the performance of 4 separate digestions of the 547 bp fragment will allow the identification of the different alleles.

#### Characterization of Multiple Phenotypic Determinants

On the basis of such enzyme-specific metabolic pathways, as exemplified hereinabove, several approaches to identifying phenotypic determinants thereof have been developed in accordance with the present invention. The characterization of multiple phenotypes offers multiple applications. The determination of an individual's metabolic phenotype for a multitude of phase I (e.g. cytochrome P450) and phase II (e.g. N-acetyltransferase) metabolic enzymes allows the use of this single profile for multiple applications. If a drug is metabolized by more than one enzyme, the phenotypic status of each of the enzymes may be important for first, determining if the individual can safely ingest a given drug and second, determining the optimal dose for this individual if they are able to take the drug.

In addition, the knowledge of multiple phenotypes will facilitate the comparison of multiple drugs within the same class or genus, where different metabolic enzymes are

involved in the metabolism of these drugs. For example, consider an individual requiring a certain class of drug, of which there are three that are primarily prescribed. If one is metabolized by CYP1A2, one by CYP2D6 and the remaining drug by CYP3A4, and all individuals that are poor metabolizers of these drugs are at risk for toxicity, then the drug chosen for treating that individual may be determined on the basis of a phenotypic profile of that individual. If for example the individual is a poor metabolizer for CYP2D6 and CYP3A4, then the first drug metabolized by CYP1A2 may be the first drug to consider for treating the individual.

Another advantage to the determination of an individual's metabolic profile for multiple phenotypic determinants is the effect of a drug on the metabolic status of enzymes not primarily involved in its metabolism. For example, a drug may be metabolized by CYP2C9 and inhibit the activity of CYP3A4. If an individual has very low levels of CYP3A4 to begin with then this inhibition may have little effect on that individual's CYP3A4 phenotype. However, if the individual is an extensive CYP3A4 metabolizer this drug may profoundly alter the CYP3A4 metabolic status. This can cause enormous problems in the case of polypharmacy, where an individual may be taking multiple drugs, and the addition of one drug may affect the safety and efficacy of the pre-existing drug treatment(s).

Examples I-III hereinbelow exemplify selected protocols for characterizing these phenotypic determinants. In addition, Examples IV-VIII outline

respective applications and uses for metabolic phenotyping information obtained from these protocols.

The metabolic phenotype can be determined directly (by measuring enzyme activity) or indirectly (by inferring levels of enzyme activity). In general, for direct phenotyping, a probe substrate or substrates, such as those exemplified in Table 4 are administered to an individual to be phenotyped. A biological sample, such as a urine sample is subsequently collected from the individual approximately 4 hours after administering the probe substrate(s). The urine sample is analyzed according to a ligand binding technology.

#### Ligand-Binding Assays

The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of both the analytical immunoassay in solution and the immunosensor on solid-state interfaces. The underlying fundamental concept of these analytical methods as ligand assays is based on the observation of the products of the ligand-binding reaction between the target analyte and a highly specific binding reagent.

The development of immunoassay technology is a success story especially for the clinical laboratory and still continues to be a vibrant area of research. Further development and automation will expand the possibilities of immunoassay analysis in the clinical sciences. Besides this, new areas for trace analyses using immunoassay were defined in the last decade: the environmental analysis of trace substances and quality control in the food industry.

Since these applications also need a continuous monitoring mode, the idea of an immunosensor as a continuously working heterogeneous immunoassay system, covering these features, was conceived. The immunosensor is now considered as a major development in the immunochemical field. Despite an overwhelming number of papers in this field, there are only a few commercial applications of immunosensors in clinical diagnostics. The reasons are, in part, unresolved fundamental questions relating to immobilization, orientation, and specific properties of the antibodies or antibody-related reagents on the transducer surface. In addition, a key issue is which clinical applications may benefit most from immunosensor devices in the routine medical laboratory. Only if there is consensus on the clinical utility of this new technique can the gap between the high expectations of the developer and reality be closed. Designers of immunosensor devices must be aware of the general and special needs of laboratory medicine from new analytical techniques.

A new analyzer should be simple and "rugged" for the measurement of analytes. Measurements have to be performed precisely and accurately, even under emergency conditions. The analyzer must be fully automated and capable of performing rapid measurements with turnaround times of < 1 h. Additionally, the determination of an analyte should preferably be without sample pretreatment in matrices, such as serum, plasma, urine or cerebrospinal fluid. All parameters determined with a new analyzer must meet the following criteria, which are defined in various guidelines: low imprecision, small lot-to-lot variations,

high analytical sensitivity, optimum analytical specificity and accuracy with long calibration stability and low interferences by drugs or normal and pathological sample components.

5        In the clinical laboratory, a future substitution of immunoassays by immunosensors simply depends on the superiority and versatility of the new methodology. The applicability for point-of-care testing or when they are temporarily implanted into the patient additionally  
10 depends on the reliable and accurate analysis of the desired analyte, without drift problems or matrix interferences. Due to the tremendously growing variety of developments, this review is not intended to be comprehensive. Hence, the main focus will be the  
15 description and assessment of reported clinical applications of immunosensors. For a more thorough understanding, we refer to several excellent reviews in the last 5 years on technical aspects and the application of immunosensors in various fields. Other related reviews  
20 deal with antibody engineering developments and latest immunoassay technologies.

Antibodies as bioaffinity interface for both immunoassays and immunosensors

25        It should first be clarified that the specificity for the measurement of analytes in all immunosensor systems, as in the case of immunoassays, is dependent on the application of affinity complexation agents (binding molecules). This pivotal feature is shared by both  
30 technologies. New developments in protein engineering for



immunoglobulins (antibody fragments, chimeric antibodies,. etc.) or in substituting antibodies by alternative binding components (e.g., aptamers) or structures (molecular imprinting) will, therefore, be applicable to either  
5 technology, if available. In particular, the possibilities in antibody engineering will enable changes in the affinity and fine specificity of antibodies, as well as the expression of fragments as fusion proteins coupled to reporter molecules.

#### 10 Immobilization procedures for antibodies

Antibodies have to be properly immobilized on the immunosensor surface, which is mostly part of a flow-  
15 through cell. The optimum density and adjusted (but not random) orientation of the antibodies are of paramount importance. Due to the different types of sensing surfaces, this manipulation can have benefits e.g., improvement of the reaction kinetic parameters, but also  
20 unfavorable effects (e.g., increased nonspecific binding, partly destroyed paratope). There are four different types of oriented coupling of antibodies: binding to Fc receptors such as protein A or G or recombinant ArG fusion protein on the surface; binding of other binding partners  
25 to structures, covalently linked to the Fc part of the antibody, e.g., the biotin residue on the Fc binds to surface-coated streptavidin; coupling to the solid support via an oxidized carbohydrate moiety on the C2 Fc domain; and the binding of Fab or scFv fragments to the surface of  
30 the device via a sulfhydryl group in its C-terminal region.

1 Numerous chemical reactions can be applied to the  
immobilization onto solid surfaces. Defined linkages  
between the antibody or its carbohydrate moieties and the  
solid phase material (silica, silanized silica, Ta- or Ti-  
5 oxides, plastics, sepharose, and metal films) are being  
built by glutaraldehyde, carbodiimide, succinimide ester,  
maleinimide, periodate or galactose oxidase. Moreover,  
photo-immobilization of antibodies using albumin  
derivatized with aryl diazirines as photolinker, is  
10 applicable. Physiosorption is not recommended due to the  
local instability of the layer caused by the mechanical  
stress in the flow-through cell. An exciting new method  
for antibody immobilization on a quartz surface of a  
piezoelectric sensor is based on the deposition of an  
15 ethylenediamine plasma polymerization film on the quartz  
crystal. This film is extremely thin and homogeneous,  
incorporating amino functions which may be further  
derivatized and linked to immunoglobulins, resulting in an  
orientation-controlled and highly reusable sensing  
20 surface. Another recent development is the planar-  
supported phospho-lipid bilayer (SLB), which can be formed  
on solid supports by vesicle fusion and Langmuir-Blodgett  
methods. SLBs maintain two-dimensional fluidity and  
accommodate multivalent binding between surface-bound  
25 ligands and receptor molecules in solution.

For noble metal surfaces, such as gold, in particular,  
in optical immunosensors, self-assembling monolayer (SAM)  
techniques seem to be first choice. In general, a SAM is  
built of long-chained ( $C_{12}$  and higher) *n*-alkylthiols with  
30 derivatized organic functional groups, which are easily

linked to the gold film via the thiol groups by a mechanism still not fully understood. The functional groups of the SAM crosslink with the Fc portion of the antibody (e.g., via the biotin streptavidin system, 5 whereas the self-organization of the matrix prevents the surface being subjected to nonspecific binding effects. In addition, the covalent coupling of IgG to a short-chain (e.g., thioctic or mercaptopropionic acid) SAM-modified metal surface has been shown to be an effective affinity- 10 based layer for optical immunosensors.

#### Regeneration of antibody-coated sensor surfaces

Conventional homogeneous and heterogeneous 15 immunoassays, respectively, work discontinuously. It is highly desirable, however, that immunosensor devices, applied in clinical diagnostics, are capable of quasi-continuous recording. The repeated use of disposable sensing elements may mimic a pseudocontinuous action, but 20 this is not considered here. In true immunosensors, the analyte/antigen interaction on the sensor-coated surface is reversible. With the given short incubation times in the flow-through device, the reaction between antigen and antibody is far off the equilibrium state. Fast 25 reversibility and high sensitivity are mutually exclusive of each other. Consistently, an adequate analytical sensitivity is only warranted if antibodies with increased affinity  $>10^{10} \text{ M}^{-1}$  or at least with highly improved on-rate are applied.

The regeneration of the binding sites of the antibodies bound to the immunosensor surface needs stringent procedures. Antibody regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is potentially harmful to the binding ability and may lead to a diminished lifetime of the immobilized antibodies and insidious drift problems.

Besides this, it must be considered that with the short reaction times between the antibodies and soluble analytes in the flow-through system, the cross-reactivities of the antibody applied can be increased. A highly specific recognition of the antigen is a kinetic-controlled process due to the complexity of the conformational changes in the Fab portion of the antibody upon binding of the antigen.

There are different approaches to solve the "antibody regeneration" problem: one approach is to displace the antigenic analyte by a highly concentrated solution of a related antigen with weak affinity to the surface-bound antibody. However, this depends on the availability of a suitable antigenic surrogate. This is not always feasible and is only applicable to small analytes. A second approach is to use the techniques of antibody engineering to improve the chemical stability of antibodies as whole molecules or as Fab fragments. The phage display technique is such a powerful tool. This can be helpful in the selection of antibody fragments with improved stability. Libraries of mutants of single-chain Fv fragments (scFv), comprising the variable regions of the L and H chains, joined by a peptide linker are generated by a combination

of site-directed and random mutagenesis. The selection can be carried out under different physical or chemical pressures to produce thermodynamically more stable scFv mutants. An interesting third approach is a pseudo-regenerating procedure for immunosensors. An amperometric sensor is coated with a conducting immunocomposite, formed by a mixture of specific antibody with methacrylate monomer and graphite. After polymerization, the device is ready for use. Repeated measurements became possible if the polymer is polished thoroughly with abrasive paper. These notes do not apply to immunosensors with a competitive configuration, in which antigenic compounds and not antibodies are surface-immobilized.

- 15 Alternative analyte-binding compounds for immunosensor applications

#### Aptamers

- Aptamers are single-stranded DNA or RNA oligonucleotide sequences with the capacity to recognize various target molecules with high affinity and specificity. These ligand-binding oligonucleotides mimic properties of antibodies in a variety of diagnostic formats. They are folded into unique overall shapes to form intricate binding furrows for the target structure. Aptamers are identified by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers may have advantages over antibodies in the ease of depositing them on sensing surfaces. Moreover, due to the highly reproducible synthetic approach in any quantities, albeit the affinity

constants are consistently lower than those of antibodies and the stability of these compounds is still questionable, they may be particularly useful for diagnostic applications in complex biological matrices.

5 The aptamer-based schemes are still in their infancy and it is expected that modified nuclease-resistant RNA and DNA aptamers will soon be available for a variety of therapeutic and diagnostic formats. The potential of aptamers for use in biosensors has been outlined in the  
10 design of a fiber-optic biosensor using an anti-thrombin DNA aptamer, immobilized on the surface of silica microspheres and distributed into microwells on the distal tip of the imaging fiber. With this device, the determination of thrombin at low concentration was  
15 possible. Exciting new possibilities are evolving by the introduction of signaling aptamers with ligand-dependent changes in signaling characteristics and catalytically active so-called "apta-zymes" which would allow the direct transduction of molecular recognition to catalysis.

20

#### Anticalins

Lipocalins constitute a family of proteins for storage or transport of hydrophobic and/or chemically sensitive organic compounds. The retinol-binding protein is an  
25 example in human physiology. It has been demonstrated that the bilin-binding protein, a member of the lipocalin family and originating from the butterfly *Pieris brassicae*, can be structurally reshaped in order to specifically complex potential antigens, such as  
30 digoxigenin, which was given as an example. These binding

proteins share a conserved  $\beta$ -barrel, which is made of eight antiparallel  $\beta$ -strands, winding around a central core. At the wider end of the conical structure, these strands are connected in a pairwise manner by four loops that form the ligand binding site. The lipocalin scaffold can be employed for the construction of so-called "anticalins", which provide a promising alternative to recombinant antibody fragments. This is made by subjecting various amino acid residues, distributed across the four loops, to targeted random mutagenesis. It remains to be shown that this class of proteins is applicable in diagnostic assays and in immunosensors. Critical points that still need to be defined include the synthesis and stability of the anticalins, the magnitude of the affinity constants, and the versatility for being crafted against the large variety of ligands.

#### Molecular imprinting techniques

This is a technique that is based on the preparation of polymeric sorbents which are selectivity predetermined for a particular substance, or group of structural analogs. Functional and cross-linking monomers of plastic materials, such as methacrylics and styrenes, are allowed to interact with a templating ligand to create low-energy interactions. Subsequently, polymerization is induced. During this process, the molecule of interest is entrapped within the polymer either by a noncovalent, self-assembling approach, or by a reversible, covalent approach. After stopping the polymerization, the template molecule is washed out. The resultant imprint of the

template is maintained in the rigid polymer and possesses a steric (size, shape) and chemical (special arrangement of complementary functionality) memory for the template. The molecularly imprinted polymer (MIP) can bind the  
5 template (= analyte) with a specificity similar to that of the antigen-antibody interaction.

Besides the main applications in solid-phase extraction and chromatography, molecularly imprinted polymers have already been employed as nonbiological  
10 alternatives to antibodies in competitive binding assays. A series of applications for analytes, such as cyclosporin A, atrazine, cortisol, 17b-estradiol, theophylline, diazepam, morphine, and S-propranolol, suggests that molecular imprinting is a promising technique for  
15 immunoassays and immunosensors.

## Immunoassay and immunosensor technologies

### Immunoassays

20 Immunoassays use antibodies or antibody-related reagents for the determination of sample analytes. This analytical tool has experienced an evolutionary history since 1959, when Berson and Yalow first described the radioimmunoassay (RIA) principle. In the RIA, a fixed and  
25 limited amount of antibody is reacted with a fixed and limited amount of radiolabeled antigen tracer and a variable concentration of the analyte. The selectivity of the ligand-binding of antibodies allows these biomolecules to be employed in analytical methods that are highly  
30 specific even in complex biological matrices, such as blood, plasma, or urine. By combining the selectivity of



antibody-analyte interactions with the vast array of antibodies preformed in immunization processes of host animals and the availability of numerous readily detectable labels radioisotopes, enzymatically or electrochemically induced adsorbance or fluorescence or chemi-luminescence, immunoassays can be designed for a wide variety of analytes while with extraordinarily low detection limits.

#### 10 Biosensors and immunosensors

A biosensor is an analytical device that integrates a biological element on a solid-state surface, enabling a reversible biospecific interaction with the analyte, and a signal transducer. The biological element is a layer of molecules qualified for biorecognition, such as enzymes, receptors, peptides, single-stranded DNA, or even living cells. If antibodies or antibody fragments are applied as a biological element the device is called an immunosensor. Compared to conventional analytical instruments, biosensors are characterized by an integrated structure of these two components. Many devices are connected with a flow-through cell, enabling a flow-injection analysis (FIA) mode of operation. Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems.

There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to

monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

Biosensors are extensively used as diagnostic tools, predominately in point-of-care testing. Probably the most successful commercialization of biosensors today is the *in vitro* near patient measurement of capillary glucose using various hand-held systems with disposable reagent cartridges.

#### Immunosensor principles

The general immunosensor design is depicted in Fig. 10. There are four types of immunosensor detection devices: electrochemical (potentiometric, amperometric or conductometric/capacitative), optical, microgravimetric, and thermometric. All types can either be run as direct nonlabeled or as indirect labeled immunosensors. The direct sensors are able to detect the physical changes during the immune complex formation, whereas the indirect sensors use signal-generating labels which allow more sensitive and versatile detection modes when incorporated into the complex.

There is a great variety of different labels which have been applied in indirect immunosensors. In principle they are the same labels as used in immunoassays. Among the most valuable labels are enzymes such as peroxidase, glucose oxidase, alkaline phosphatase (AP), catalase or luciferase, electroactive compounds such as ferrocene or

In<sup>2+</sup> salts, and a series of fluorescent labels (including rhodamine, fluorescein, Cy5, ruthenium diimine complexes, and phosphorescent porphyrin dyes). In particular, laser-induced fluorometric resonance energy transfer between two fluorophores offers methodological advantages and can be extended to fiberoptic sensing.

Although indirect immunosensors are highly sensitive due to the analytical characteristics of the label applied, the concept of a direct sensor device is still fascinating and represents a true alternative development to immunoassay systems. Its potential simplicity holds multiple advantages, making immunosensors progressive and future directed.

The present invention will be illustrated using the following examples, which are not to be seen as limiting in any way. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

#### Electrochemical sensors

*Potentiometric immunosensors.* The Nernst equation provides the fundamental principle of all potentiometric transducers. According to this equation, potential changes are logarithmically proportional to the specific ion activity. Potentiometric transducer electrodes, capable of measuring surface potential alterations at near-zero

current flow, are being constructed by applying the following methodologies.

*Transmembrane potential.* This transducer principle is based on the accumulation of a potential across a sensing membrane. Ion-selective electrodes (ISE) use ion-selective membranes which generate a charge separation between the sample and the sensor surface. Analogously, antigen or antibody immobilized on the membrane binds the corresponding compound from the solution at the solid-state surface and changes the transmembrane potential.

*Electrode potential.* This transducer is similar to the transmembrane potential sensor. An electrode by itself, however, is the surface for the immunocomplex building, changing the electrode potential in relation to the concentration of the analyte.

*Field-effect transistor (FET).* The FET is a semiconductor device used for monitoring of charges at the surface of an electrode, which have been built up on its metal gate between the so-called source and drain electrodes. The surface potential varies with the analyte concentration. The integration of an ISE with FET is realized in the ion-selective field-effect transistor (ISFET). This technique can also be applied to immunosensors.

An advantage of potentiometric sensors is the simplicity of operation, which can be used for automation, and the small size of the solid-state FET sensors. All potentiometric methods, however, are still suffering from major problems of sensitivity, being inferior to amperometric transducers and nonspecific effects of

binding or signaling influences from other ions present in the sample. Especially, the signal-to-noise ratio causes analytical problems, which are difficult to circumvent. Thus, a trend away from these techniques has been observed in the last few years. However, the ISFET may be seen as a candidate for ultrasensitive clinical immunosensor applications, in particular, when the novel concept of differential ISFET-based measurement of the zeta potential is used. The streaming potential is a potential difference in flow direction, caused by the flow of excess ions resulting from a local distortion of the charge balance. The zeta potential, directly correlated to the streaming potential, reflects the potential changes in the diffuse outer layer at the solid-liquid interface. It efficiently reacts to protein accumulations onto sensor surfaces and, thus, is suitable for detecting immunocomplex reactions.

#### *Amperometric immunosensors.*

Amperometric immunosensors are designed to measure a current flow generated by an electrochemical reaction at constant voltage. There are only few applications available for direct sensing, since most protein analytes are not intrinsically able to act as redox partners in an electrochemical reaction. Therefore, electrochemically active labels directly or as products of an enzymatic reaction are needed for the electrochemical reaction of the analyte at the sensing electrode. Oxygen and  $H_2O_2$  electrodes are the most popular. An oxygen electrode consists of an electrolyte-bearing chamber with a sensing Pt cathode, polarized at 0.7 V, and an Ag/AgCl reference

electrode. The chamber is gas-permeable, covered by an O<sub>2</sub>-pervious membrane.

Besides oxygen, generated by catalase from H<sub>2</sub>O<sub>2</sub> there are other amperometrically detectable compounds, such as ferrocene derivatives or In<sup>2+</sup> salts. A novel approach is the use of the redox polymer [PVP-Os(bipyridyl)<sub>2</sub>Cl ], which is coimmobilized with specific antibodies. Additionally, there are examples for enzymes with electrochemically active products. AP, for example, catalyzes the hydrolysis of phenyl phosphate or *p*-aminophenyl phosphate (4-APP) compounds, which result in electrochemically active phenol or *p*-aminophenol. Furthermore, enzymes, such as horseradish peroxidase (HRP), glucose oxidase, glucose-6-phosphate dehydrogenase, with subsequent amperometrical oxidation of NADH and others, have also been successfully applied as labels.

The main disadvantage for amperometric immunosensors of having an indirect sensing system, however, is compensated for by an excellent sensitivity. This is due to a linear analyte concentration range compared to a logarithmic relationship in potentiometric systems. Special attention must be directed to the system-inherent transport rate limitations for redox partners on the electrode surface.

25

#### *Conductometric and capacitive immunosensors.*

These immunosensor transducers measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical enzymatic reactions which specifically generate or consume ions. The capacitance

30

changes are measured using an electrochemical system, in which the bioactive element is immobilized onto a pair of noble metal, mostly Au or Pt, electrodes. There are only few clinical applications available, as the high ionic strength of biological matrices makes it difficult to record the relatively small net conductivity changes caused by the signaling reaction. To circumvent this problem, recently, an ion-channel conductance immunosensor, mimicking biological sensory functions, was developed. The basis of this technique is the fact that the conductance of a population of molecular ion channels, built of tethered gramicidin A and aligned across a lipid bilayer membrane, is changed by the antibody-antigen binding event. Different applications using various antibodies, linked to the ion-channel complex, are given.

Another approach is the measurement of changes of the surface conductivity. For example, a conductometric immunosensor for the determination of methamphetamine (MA) in urine was recently developed. Anti-MA antibodies were immobilized onto the surface of a pair of platinum electrodes. The immunocomplex formation caused a decrease in the conductivity between the electrodes.

The measurement of the reciprocal capacitance, performed at alternating voltage, is advantageous compared to conductometric devices, and serves two purposes. The first is to test the insulating monolayer on the sensor noble metal surface. Self-assembling monolayers, have insulating properties. Besides this, they prevent the immunosensor from being affected by nonspecific binding phenomena. Even minor desorption of the monolayer results

in an essential increase in capacitance. Thus, the actual quality of the device can be checked. The second application is the measurement of changes in the effective dielectric thickness of the insulating layer during antigen binding, when antibodies are linked to the alkylthiol layer. Of course, this is on condition that the v-substitution of the alkylthiol monolayer does not compromise the insulation. Hence, a marked decrease of the electrical capacitance is observed and is used to quantitate the analyte. The destructive influence of lateral diffusion on nanostructured monolayers is prevented by use of the spreader-bar technique.

#### Optical sensors

Optical immunosensors are most popular for bioanalysis and are today's largest group of transducers. This is due to the advantages of applying visible radiation compared to other transducer techniques. Additional benefits are the nondestructive operation mode and the rapid signal generation and reading. In particular, the introduction of fiber bundle optics ("optodes") as optical waveguides and sophisticated optoelectronics offers increased versatility of these analytical devices for clinical applications.

Changes in adsorption, fluorescence, luminescence, scatter or refractive index (RI) occur when light is reflected at sensing surfaces. These informations are the physical basis for optical sensor techniques. Usually, applied detectors are photodiodes or photomultipliers.

There are numerous applications of either direct label-free optical detection of the immunological reaction, of labeled immunospecies, or of the products of



enzymatic reactions. Most labels are fluorescent, but bio- and chemiluminescence species are also possible. It is worth mentioning that the label-free evanescence wave-related sensors explicitly represent an elegant methodology, which is a valuable alternative to sophisticated immunoassays. Nevertheless, label-free systems are prone to unsolved problems, such as nonspecific binding effects and poor analytical sensitivity to analytes with low molecular weight.

Kubitschko et al. noted that despite the efforts, all immunosensors are still one magnitude less sensitive than commercial immunoassays for determining analytes in human serum, particularly those with low molecular weight. They claim the use of mass labels, such as latex particles, in order to enhance the signal. The authors demonstrated the optimization of a nanoparticle enhanced bidiffractive grating coupler immunosensor for the detection of thyroid-stimulating hormone (TSH, MW 28,000 Da). The excellent performance characteristics of this sensor clearly showed how future devices should work. The problem of unspecific binding, however, can also be controlled by applying a reference sensing region on the chip.

#### *Total internal reflection spectroscopy (TIRS).*

The common principle of the following analytical devices is that in an optical sensor with two materials with different refractive indices (RI), total internal reflection occurs at a certain angle of the light beam being directed through the layer with the higher RI towards the sensing interface. By this, an evanescence

wave is generated in the material with the lower RI. This wave, being an electrical vector of the wavelength of the incident light beam, penetrates further into the medium with exponentially attenuated amplitude. Biomolecules attached in that portion of the medium will interact inevitably with the evanescent wave and, therefore, lead to a distinctive diminution of the reflected light. The resolution is directly proportional to the length of interaction. Infrared spectrosopes, measuring attenuated total reflectance, are commonly built in the Kretschmann configuration: an optically absorbing film at the sensor's surface enables the measurement of the attenuated light intensity as a function of the wavelength of the incident beam. For total internal reflection fluorescence (TIRF), analytics benefit from the fact that incident light excites molecules with fluorescence characteristics near the sensor surface creating a fluorescent evanescent wave. The emerging fluorescence is finally detected. The technique has been developed mainly for an optical detection of fluorescence-labeled antibodies or antigens. In the latter case, the fluorescence capillary fill device (FCFD) technique is worth mentioning. The FCFD is designed by using a planar optical waveguide and a glass plate separated from each other by a capillary gap. Fluorophore-labeled antigen is attached on the surface of the glass plate, whereas antibodies are immobilized on the surface of the optical waveguide.

Another phenomenon, the optical diffraction, is used by the optical biosensor assay (OBA<sup>TM</sup>) system: biomolecules are attached to the surface of a silanized wafer. The

protein-coated surface is illuminated through a photo mask to create distinct periodic areas of active and inactive protein. Upon illumination with laser light, the diffraction grating caused by the ligand-binding process diffracts the incident light. An analyte-free negative sample does not result in diffraction because no antigen-antibody binding occurred creating the diffraction grating. The presence or absence of a diffraction signal differentiates between positive and negative samples. The intensity of the signal provides a quantitative measure of the analyte concentration.

#### Ellipsometry

If linearly polarized light of known orientation is reflected at oblique incidence from a surface, the reflected light is elliptically polarized. The shape and orientation of the ellipse depend on the angle of incidence, the direction of the polarization of the incident light, and the reflection properties of the surface. On adsorption of biomolecules onto a planar solid surface, phase and amplitude of the reflected light are altered and can be recorded by ellipsometric techniques. These changes in the polarization of the light are due to the alterations of the RI and the coating thickness. There are only few applications, such as the study of a cholera toxin-ganglioside GM1 receptor-ligand reaction, which were carried out using an ellipsometer.

*Optical dielectric waveguides.*

Optical waveguides are glass, quartz or polymer films or fibers made of high RI material embedded between or in lower index dielectric materials. If a linearly polarized helium-neon laser light wave, introduced into the high index film or fiber, arrives at the boundary at an angle which is greater than the critical angle of total reflection, it is confined inside the waveguide. Similar to surface plasmon resonance, an evanescent field develops at the sensor's surface. In this case, however, the evanescent field is generated by the excitation of the light itself in the dielectric layer. Most of the laser light is transmitted into the device and multiple reflections occur as it travels through the medium if a bioactive substance is placed over the surface. Some of the light, however, penetrates the biolayer. This light is reflected back into the waveguide with a shift in phase interfering with the transmitted light. Thus, changes in properties of the biolayer can be followed by detecting the changes in interference.

Waveguides are often made in the form of fibers. These fiber-optic waveguide systems offer advantages for sensors when being used for hazardous analysis. Planar waveguide systems are also applicable for interferometers. They use laser light directed towards the surface of the waveguide with the attached biomolecules, which is subsequently split into two partial electrical (TE) and magnetic <sup>TM</sup> fieldwaves, perpendicular to each other. The interaction with the sample surface changes the relative phase between TE and TM by the different RI and surface thickness

values. Various configurations, such as the Fabry-Perot monomode channel interferometer, the Mach-Zehnder interferometer or the related two-mode thin-film waveguide difference interferometer, have been successfully established.

Another technique uses thin corrugations etched into the surface of a waveguide. This grating coupler device allows the measurement of the coupling angle of either the input or output laser beam. Both beams are correlated to the RI within the evanescent field at the sensor's surface. Recently, a long-period grating fiber immunosensor has proven to be sensitive (enabling analyses down to the nanomolar range) and reproducible. Grating couplers are also used for optical waveguide lightmode spectroscopy (OWLS). The basic principle of the OWLS method is that linearly polarized light is coupled by a diffraction grating into the waveguide layer. The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the RI of the medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the light, the mode spectrum is obtained from which the effective RIs are calculated for both TE and TM.

#### *Surface plasmon resonance (SPR).*

Among the different detection systems, SPR is the most popular one. There are two leading systems on the market: the BIAcore™ systems from Biacore (Uppsala, Sweden) and

the IAsys<sup>TM</sup> from Fisons Applied Sensor Technology (Cambridge, UK). Other systems with small market positions are the BIOS-1 from Artificial Sensing Instruments (Switzerland), the SPR-20 from Denki Kagaku Keiki (Japan),  
5 the SPEETA from Texas Instruments (USA), the IBIS from Windsor Scientific (UK) and the DPX from Quantech (USA). The first two commercial evanescence-wave devices are widespread in research laboratories due to the sophisticated apparatus and userfriendly control software.

10 The BIAcore<sup>TM</sup>, however, has the biggest market position.

The general principle of SPR measurement 80 is depicted in Fig. 11. Polarized light is directed from a layer of high RI towards a layer with low RI to result in total internal reflection. The sample is attached to the  
15 layer of low RI. At the interface between the two different media, a thin approximately 50 nm gold film is interposed. Although light does not propagate into the low RI medium, the interfacial intensity is not equal to zero. The physical requirement of continuity across the  
20 interface is the reason for exciting the surface electrons "plasmons" in the metal film by the light energy. As a result, the electrons start oscillating. This produces an exponentially decaying evanescent wave penetrating a defined distance into the low RI medium, which is  
25 accountable for a characteristic decrease in the intensity of the reflected light. Hence, a direct insight in changes of the RI at the surface interface is made possible by monitoring the intensity and the resonance angle of the reflected light, caused by the biospecific interactions

which took place there. Whereas in the BIAcore™ system, the light affects the sensing layer only once, there are several propagation contacts in the IAsys™ due to the device's resonant mirror configuration. The BIAcore™ SPR apparatus is characterized by a sensitive measurement of changes of the RI when polarized laser light is reflected at the carboxy-methylated dextranactivated device interface. The IAsys™ SPR device also uses a carboxy-methylated dextran-activated surface. Its dextran layer, however, is not attached to a gold surface, but to titanium, which forms a high refractive dielectric resonant layer. The glass prism is not attached tightly on the opposite side of the titanium layer, making space for an interposed silica layer of low RI. By this layer, the laser light beam couples into the resonant layer via the evanescent field. Therefore, the IAsys™ is seen as a combination of SPR resonant mirror with waveguide technology. As a result, no decrease in the reflected light intensity at resonance is observed in this system. The specific signal is the change in the phase of the reflected polarized light.

Differential SPR, a novel modification of a SPR immunosensor, improves further the sensitivity of the sensor by applying a modulation of the angle of light incidence. The reflectance curve is measured with a lock-in amplifier and recorded in the first and second derivative.

Light is directed from a prism with a RI towards a layer with low RI, resulting in total internal reflection.

Although light does not propagate into the medium, the interfacial intensity is not equal to zero. Physical requirements of continuity across the interface are the cause of excitation of surface plasmons in the metal film by the light energy, causing them to oscillate. This produces an exponential evanescent decaying, which penetrates a defined distance into the low-index medium and results in a characteristic decrease in reflected light intensity.

#### Microgravimetric sensors

A direct measurement of mass changes induced by the forming of antigen/antibody complexes is also enabled by acoustic sensors. The principle of operation is based on the propagation of acoustic shear waves in the substrate of the sensor. Phase and velocity of the acoustic wave are influenced by the specific adsorption of antibody molecules onto the antigen-coated sensor surface.

Piezoelectric materials, such as quartz ( $\text{SiO}_2$ ), zinc oxide ( $\text{ZnO}$ ) or others resonate mechanically at a specific ultrasonic frequency in the order of tens of megahertz when excited in an oscillating electrical field. The resonant frequency is determined by the distance between the electrodes on both sides of the quartz plate, which is equal to the thickness of the plate and the velocity of the acoustic wave within the quartz material. In other words, electromagnetic energy is converted into acoustic energy, whereby piezoelectricity is associated with the electrical polarization of materials with anisotropic crystal structure. The most applied technique for



monitoring the acoustic wave operation is the oscillation method. This means a configuration in which the device constitutes the frequency-controlling element of a circuit. The oscillation method measures the series  
5 resonant frequency of the resonating sensor.

The microgravimetric sensor devices are divided into quartz crystal microbalance (QCM) devices applying a thickness-shear mode (TSM), and devices applying a surface acoustic wave (SAW) detection principle. These sensors  
10 have reached considerable technical sophistication.

Additional bioanalytical application devices include the flexural plate wave (FPW), the shear horizontal acoustic plate (SH-APM), the surface transverse wave (STW) and the thin-rod acoustic wave (TRAW)

15 There are considerable similarities between the physical principles of QCM and SPR sensors, even when there are fundamental differences. Both QCM and SPR are wave-propagation phenomena and show resonance structure. The elastic QCM wave and the surface plasmon wave are  
20 nonradiative, i.e., an evanescent wave exists. Changes of physical properties within the evanescent field lead to a shift of resonance. Thus, a linear approximation of the physical relationship is allowed for immunological application in immunosensors.

25

The TSM sensor

The TSM sensor consists of an AT-cut piezoelectric crystal disc, most commonly of quartz because of its chemical stability in biological fluids and resistance to  
30 extreme temperatures. The disc is attached to two metal

electrodes on opposite sides for the application of the oscillating electric field. The TSM is run in a range of 5-20 MHz. The schematic design of a typical TSM device shown in Fig. 12. Advantages are, besides the chemical inertness, the low cost of the devices and the reliable quality of the mass-produced quartz discs. Major drawbacks of the system are the insensitivity for analytes with a molecular weight  $>1000$  Da, and, as seen in all label-free immunosensor systems, nonspecific binding interferences. Nonspecific binding effects are hard to distinguish from authentic binding events due to the fact that no reference line can be placed in the sensor device. For a SH-APM device, however, by appropriately selecting the device frequency, these spurious responses can be suppressed. This sensor is applicable for measurements in human serum matrix.

One of the first applications of TSM technology was an immunosensor for human immunodeficiency virus (HIV) serology. This sensor was realized by immobilizing recombinant viral peptides on the surface of the transducer and by detecting anti-HIV antibodies directly in human sera.

#### *The SAW sensor.*

SAW sensors use thick ST-cut quartz discs and interdigitated metal electrode arrays that generate acoustic Rayleigh waves in both directions from the interdigital electrodes, their transmission being attenuated by surface-attached biomolecules. The oscillation frequency of a SAW sensor ranges from 30 to

500 MHz. The operation of SAW immunosensors with biological samples is compromised by the fact that the surface wave is considerably attenuated in the liquid phase. Thus, the domain of this technique is most likely restricted to gas phase operations.

The present invention is exemplified as an ELISA as described hereinbelow for corresponding probe substrate and or metabolites and the molar ratios thereof calculated to reveal the individual phenotypes.

**Table 4**  
**Examples of Enzymes and Corresponding Probes Drugs**

Enzyme	Probe substrate
NAT1	p-aminosalicylic acid
NAT2	Caffeine
CYP1A2	Caffeine
CYP2A6	Coumarin
CYP2C9	Diclofenac
CYP2C19	s-ibuprofen
CYP2D6	Dextromethorphan
CYP2E1	Chlorzoxazone
CYP3A4	Midazolam

In Example I, a detailed description of the synthesis of probe substrate and metabolite derivatives and the ELISA development for N-acetyltransferase (NAT2) and CYP1A2 are illustrated. The materials and methods, and the overall general process described for the development of the NAT2 and CYP1A2 ELISA method and kit for metabolic phenotyping can be and will be applied to the development

of the metabolic phenotyping ELISA kits for other metabolic enzymes including NAT1, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, as well as a multi-determinant metabolic phenotyping system and method.

5 While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention  
10 encompassed by the appended claims.

#### EXAMPLE I

##### Determination of Phenotypic Determinants by ELISA

##### NAT2

15 Different probe substrates can be used to determine the NAT2 phenotype (Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, 47:470-477; Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, 49:648-657). In accordance with the present invention caffeine is the preferred probe  
20 because it is widely consumed and relatively safe (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, 53:503-514). In studies involving this probe, the phenotype has been generally determined from ratios of the caffeine metabolites 5-acetamino-6-amino-1-methyluracil (AAMU) or  
25 5-acetamino-6-formylamino-1-methyluracil (AFMU) and 1-methylxanthine (1X). In these studies, the subjects are given an oral dose of a caffeine-containing substance, and the urinary concentrations of the target metabolites determined by HPLC (Kilbane, A.J. et al. (1990) *Clin.*

*Pharmacol. Ther.*, 47:470-477; Tang, B-K. et al. (1991)  
*Clin. Pharmacol. Ther.*, 49:648-657) or CE (Lloyd, D. et  
al. (1992) *J. Chrom.*, 578:283-291).

The number of clinical protocols requiring the  
5 determination of NAT2 phenotypes is rapidly increasing and  
in accordance with the present invention, an enzyme linked  
immunosorbent assay (ELISA) was developed for use in these  
studies (Wong, P., Leyland-Jones, B., and Wainer, I.W.  
(1995) *J. Pharm. Biomed. Anal.*, 13:1079-1086). ELISAs have  
10 been successfully applied in the determination of low  
amounts of drugs and other antigenic components in plasma  
and urine samples, involve no extraction steps and are  
simple to carry out.

In accordance with the present invention, antibodies  
15 were raised in animals against two caffeine metabolites [5-  
acetamino-6-amino-1-methyluracil (AAMU) or 5-acetamino-6-  
formylamino-1-methyluracil (AFMU), and 1-methyl xanthine  
(1X)] present in urine samples of an individual collected  
after drinking coffee. Their ratio provides a  
20 determination of an individual's N-acetylation (NAT2)  
phenotype. Subsequently, there was developed a competitive  
antigen enzyme linked immunosorbent assay (ELISA) for  
measuring this ratio using these antibodies.

The antibodies of the present invention can be either  
25 polyclonal antibodies or monoclonal antibodies raised  
against two different metabolites of caffeine, which allow  
the measurement of the molar ratio of these metabolites.  
In accordance with the present invention, the molar ratio  
of caffeine metabolites is used to determine the  
30 acetylation phenotype of the individual as follows.

Individuals with a ratio less than 1.80 are slow acetylators.

## Materials and Methods

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### Materials

Cyanomethylester, isobutyl chloroformate, dimethylsulfate, sodium methoxide, 95% pure, and tributylamine were purchased from Aldrich (Milwaukee, WI, USA); horse radish peroxidase was purchased from Boehringer Mannheim (Montreal, Que., Canada); Corning easy wash polystyrene microtiter plates were bought from Canlab (Montreal, Que., Canada); o-methylisourea hydrochloride was obtained from Lancaster Laboratories (Windham, NH, USA); alkaline phosphatase conjugated to goat anti-rabbit IgGs was from Pierce Chemical Co. (Rockford, IL, USA); bovine serum albumin fraction V initial fractionation by cold alcohol precipitation (BSA), complete and incomplete Freund's adjuvants, diethanolamine, 1-methylxanthine, p-nitrophenol phosphate disodium salt, o-phenylenediamine hydrochloride; porcine skin gelatin, rabbit serum albumin (RSA); Sephadex™ G25 fine, Tween™ 20 and ligands used for testing antibodies' cross reactivities were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Whatman™ DE52 diethylaminoethyl-cellulose was obtained from Chromatographic Specialties Inc. (Brockville, Ont., Canada). Dioxane was obtained from A&C American Chemicals Ltd. (Montreal, Que., Canada) and was refluxed over calcium hydride for 4 hours and distilled before use.

30 Other reagents used were of analytical grade.

## Synthetic procedures

The synthetic route for the production of AAMU-hemisuccinic acid (VIII) and 1-methylxanthine-8-propionic acid (IX) is presented in Fig. 13.

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## Synthesis of 2-methoxy-4-imino-6-oxo-dihydropyridine (III)

Compound III is synthesized according to the procedure of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, **90**:2272-2276) as follows. . To a 250 mL round bottom flask 12.2 g of o-methylisourea hydrochloride (110.6 mmol), 11.81 mL methylcyanoacetate (134 mmol), 12.45 g of sodium methoxide (230.5 mmol) and 80 mL of methanol are added. The suspension is stirred and refluxed for 5 hours at 68-70°C. After cooling at room temperature, the suspension is  
10 filtered through a sintered glass funnel (Pyrex, 40-60 ASTM, 60 mL), and the NaCl on the filter is washed with methanol. The filtrate is filtered by gravity through a Whatman<sup>TM</sup> no. 1 paper in a 500 mL round bottom flask, and the solvent is evaporated under reduced pressure with a  
15 rotary evaporator at 50°C. The residue is solubilized with warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel  
20 (Pyrex, 40-60 ASTM, 60 mL). The product is washed with water, acetone, and dried. The product is recrystallized with water as the solvent and using charcoal for decolorizing (activated carbon, Norit<sup>r</sup> A< 100 mesh, decolorizing). The yield is 76 %.

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Synthesis of 1-methyl-2-methoxy-4-imino-6-oxo-  
dyhydropyrimidine (IV)

Compound IV is synthesized according to the procedure  
5 of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-  
2276) as follows. . To a 250 mL round bottom flask 11g of  
compound III (77.0 mmol) and 117 mL of 1N NaOH (freshly  
prepared) are added. The solution is stirred and cooled at  
15°C, using a water bath and crushed ice. Then 11.7 mL  
10 dimethylsulfate (123.6 mmol) are added dropwise with a  
pasteur pipette over a period of 60 min. Precipitation  
eventually occurs upon the addition. The suspension is  
stirred at 15°C for 3 hours and is left at 4°C overnight.  
The product is recovered by filtration under vacuum  
15 through a sintered glass funnel (Pyrex, 40-60 ASTM, 60  
mL). The yield is 38 %.

Synthesis of 1-methyl-4-iminouracil (V)

Compound V is synthesized according to the procedure  
20 of Pfeiderer (Pfeilderer, W. (1957) *Chem. Ber.*, 90:2272-  
2276) as follows. . To a 250 mL round bottom flask 11.26  
g of compound IV (72.6 mmol) and 138 mL 12 N HCl are  
added, and the suspension is stirred at room temperature  
for 16-20 hours. The suspension is cooled on crushed ice,  
25 the product is recovered by filtration under vacuum  
through a sintered glass funnel (Pyrex, 40-60 ASTM, 60  
mL). The product is washed with water at 4°C, using a  
pasteur pipette, until the pH of filtrate is around 4



(about 150 mL). The product is washed with acetone and dried. The yield is 73 %.

#### Synthesis of 1-methyl-4-imino-5-nitrouracil (VI)

- 5 Compound VI is synthesized according to the procedure of Lespagnol et al (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. . To a 250 mL round bottom flask 6.5 g of compound V (46 mmol) and 70 mL of water are added. The suspension is stirred and refluxed at 100°C. A
- 10 solution of 6.5 g sodium nitrite (93.6 mmol) dissolved in 10 mL water is added gradually to the reaction mixture with a pasteur pipette. Then 48 mL of glacial acetic acid is added with a pasteur pipette. Upon addition, precipitation occurs and the suspension becomes purple.
- 15 The suspension is stirred and heated for an additional 5 min., and cooled at room temperature and then on crushed ice. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 10-15 ASTM, 60 mL). It is washed with water at 4 °C to remove acetic acid
- 20 and then with acetone. Last traces of acetic acid and acetone are removed under a high vacuum. The yield is 59 %.

#### Synthesis of 1-methyl-4,5-diaminouracil (VII)

- 25 Compound VII is synthesized by the procedure of Lespagnol et al. (Lespagnol, A. et al.(1970) *Chim. Ther.*, 5:321-326) as follows. To a 100 mL round bottom flask 2 g of compound VI (11.7mmol) and 25 mL water are added. The suspension is stirred and heated in an oil bath at 60°C.
- 30 Sodium hydrosulfite (88%) is gradually added (40.4 mmol),

using a spatula, until the purple color disappears (approximately 5 g or 24.3 mmol). The suspension is heated for an additional 15 min. The suspension is cooled on crushed ice and left at 4°C overnight. The product is recovered by filtration under vacuum through a sintered glass funnel (Pyrex, 30-40 ASTM, 15 mL). The product is washed with water and acetone, and dried. The last traces of acetone are removed under a high vacuum. The yield is 59%.

#### 10 Synthesis of AAMU-hemisuccinic acid (VIII)

Compound VIII is synthesized as follows. To a 20 mL beaker 0.30 g of compound VII (1.92 mmol) and 5 mL water are added. The suspension is stirred and the pH is adjusted between 8 to 9 with a 3N NaOH solution. Then 0.33 g succinic anhydride (3.3 mmol) is added to the resulting solution, and the mixture is stirred until the succinic anhydride is dissolved. During this process, the pH of the solution is maintained between 8 and 9. The reaction is completed when all the succinic anhydride is dissolved and the pH remains above 8. The hemisuccinate is precipitated by acidification to pH 0.5 with 12N HCl. The product is recovered by filtration on a Whatman™ No. 1 paper, and washed with water to remove HCl. It is then washed with acetone and dried.

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#### Other AAMU or AFMU derivatives

The derivatives shown in Figs. 14 and 15 can also be used for raising antibodies against AAMU or AFMU that can be used for measuring the concentrations of these caffeine metabolites in urine samples.

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### Synthesis of 1-methylxanthine-8-propionic acid (IX)

This product is synthesized according to a modified procedure of Lespagnol et al. (Lespagnol, A. et al. (1970) *Chim. Ther.*, 5:321-326) as follows. A 0.2 g sample of compound VIII (0.78 mmol) is dissolved in 2-3 mL of a 15% NaOH solution. The resulting solution is stirred at 100°C until all of the solvent is evaporated, and is then maintained at this temperature for an additional 5 min. The resulting solid is cooled at room temperature, and dissolved in 10 mL water. The product is precipitated by acidification to pH 2.8 with 12 N HCl. After cooling at 4°C for 2.5 hours, the product is recovered by filtration on a Whatman<sup>TM</sup> No. 1 paper, washed with water and acetone, and dried. It is recrystallized from water-methanol (20:80, v/v), using charcoal to decolorize the solution.

### Other derivatives of 1X

The other derivatives of 1X, shown in Figs. 16 and 17, can also be used for raising antibodies against 1X and thereby to allow the development of an ELISA for measuring 1X concentration in urine samples.

### Synthesis of AAMU

AAMU is synthesized from compound VII according to the procedure of Fink et al (Fink, K. et al. (1964) *J. Biol. Chem.*, 249:4250-4256) as follows. To a 100 mL round bottom flask 1.08 g of compound VII (6.9 mmol) and 20 mL acetic acid anhydride were added. The suspension is stirred and refluxed at 160-165 °C for 6 min. After cooling at room temperature, the suspension is filtered under vacuum

## 5 NMR spectroscopy

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and the resulting solution is poured in one portion to the flask containing the first solution. The solution is stirred at 10-12°C for 3 hours and dialyzed against 1 liter of water for 2 days at room temperature, with water changed twice a day. The protein concentration of the conjugates and the amounts of moles of AAMU or 1X incorporated per mole of BSA or RSA is determined by methods described below. The products are stored as 1 mL aliquots at -20°C

Protein determination by the method of Lowry et al.  
(Lowry, O.H. et al. (1951) *J. Biol. Chem.*, 193:265-275)

#### A) Solutions

Solution A: 2 g Na<sub>2</sub>CO<sub>3</sub> is dissolved in 50 mL water, 10 mL of 10 % SDS and 10 mL 1 NaOH, water is added to 100 mL. Freshly prepared.

Solution B: 1 % NaK Tartrate

Solution C: 1 % CuSO<sub>4</sub>.5H<sub>2</sub>O

Solution D: 1 N phenol (freshly prepared): 3 mL Folin & Ciocalteu's phenol reagent (2.0 N) and 3 mL water.

Solution F: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared.

BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction V)/100 mL.

#### B) Assay

Standard curve	Tubes # (13 x 100 mm)						
Solution	1	2	3	4	5	6	7
BSA $\mu$ L)	0	10	15	20	30	40	50
Water $\mu$ L)	200	190	185	180	170	160	150
Solution F (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

The solutions are vortexed and left 10 min at room temperature.

Solution D (μL)                      200    200    200    200    200    200    200

5        The solutions are vortexed and left at room temperature for 1 hour.

The absorbance of each solution is read at 750 nm using water as the blank.

**Unknown**

Solution	D.F. <sup>a</sup>	Tube # (13 x 100 mm)		
		1	2	3
Unknown (μL)		x	x	x
Water (μL)		y	y	y
		$x + y = 200 \mu\text{L}$		
Solution F (mL)		2.0	2.0	2.0

15        The solutions are vortexed and left 10 min at room temperature.

Solution D (μL)                      200    200    200

20        The solutions are vortexed and left 1 hour at room temperature.

The absorbance of each solution is read at 750 nm using water as the blank.

25        The protein concentration is calculated using the standard curve and taking account of the dilution factor (D.F.).

a. D.F. (dilution factor). It has to be such so that the absorbance of the unknown at 750 nm is within the range of absorbance of the standards.

Method to determine the amounts of moles of AAMU or 1X incorporated per mole of BSA or RSA.

This method gives an approximate estimate. It is a useful one because it allows one to determine whether the coupling proceeded as expected.

#### A) Solutions

- 10% sodium dodecyl sulfate (SDS)
- 1% SDS solution
- 10 - 0.5 or 1 mg/mL of AAMU-BSA (or AAMU-RSA) in a 1% SDS solution (1 mL).
- 0.5 or 1 mg/mL of BSA or RSA in a 1% SDS solution (1 mL).

#### 15 B) Procedure

- The absorbance of the AAMU conjugate solution is measured at 265 nm, with 1% SDS solution as the blank.
- The absorbance of the BSA (or RSA) solution is measured at 265 nm, with 1% SDS solution as the blank.
- 20 - The amount of moles of AAMU incorporated per mole of BSA (or RSA) is calculated with this formula:

$$Y = \frac{A_{265} \text{ (AAMU-BSA)} - A_{265} \text{ (BSA)}}{\epsilon_{265} \text{ (AAMU)} \times [\text{BSA}]}$$

Where:

y is the amount of moles of AAMU/mole of BSA (or RSA);  
 $\epsilon_{265}$  (AAMU) is the extinction coefficient of AAMU =  $10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ; and

30 [BSA] = BSA (mg/mL) / 68,000/mole.

To calculate the amount of moles of 1X incorporated per mole of BSA or RSA, the same procedure is used but with this formula:

$$y = \frac{A_{252} (1X-BSA) - A_{252} (BSA)}{\epsilon_{252} (1X) \times [BSA]}$$

Where:

- 5 y is the amount of moles of 1X/mole of BSA (or RSA);  
 $\epsilon_{252}$  (AAMU) is the extinction coefficient of 1X =  $10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ; and  
[BSA] = BSA (mg/mL) / 68,000/mmmole.

Coupling of haptens to horse radish peroxidase

- 10 The AAMU derivative (VIII) and 1X derivative (IX) are conjugated to horse radish peroxidase (HRP) by the following procedure. To a 5 mL round bottom flask 31.2 mg of compound VIII (or 28.3 mg of compound IX) are added. Then 500  $\mu\text{L}$  of dioxane, freshly dried over calcium
- 15 chloride, are added. The suspension is stirred and cooled at  $10^\circ\text{C}$  using a water bath and crushed ice. Then 114  $\mu\text{L}$  tributylamine and 31  $\mu\text{L}$  of isobutyl chloroformate (recently opened or purchased) are added. The suspension is stirred for 30 min at  $10^\circ\text{C}$ . While the suspension is
- 20 stirring, a solution is prepared by dissolving 13 mg of horse radish peroxidase (HRP) in 2 mL of water. The solution is cooled at  $4^\circ\text{C}$  on crushed ice. After the 30 min stirring, 100  $\mu\text{L}$  of a 1 N NaOH solution at  $4^\circ\text{C}$  is added to the HRP solution and the alkaline HRP solution is poured
- 25 at once into the 5 mL flask. The suspension is stirred for 4 hours at  $10-12^\circ\text{C}$ . The free derivative is separated from the HRP conjugate by filtration through a Sephadex G-25<sup>TM</sup> column (1.6 x 30 cm) equilibrated and eluted with a 0.05 M sodium phosphate buffer, pH 7.5. The fractions of
- 30 1.0-1.2 mL are collected with a fraction collector.



During the elution two bands are observed: the HRP conjugate band and a light yellow band behind the HRP conjugate band. The HRP conjugate elutes between fractions 11-16. The fractions containing the HRP

5 conjugate are pooled in a 15 mL tissue culture tube with a screw cap. The HRP conjugate concentration is determined at 403 nm after diluting an aliquot (usually 50  $\mu$ L+650  $\mu$ L of buffer).

$$[\text{HRP-conjugate}] (\text{mg/mL}) = A_{403} \times 0.4 \times \text{D.F.}$$

10 The ultraviolet (UV) absorption spectrum is recorded between 320 and 220 nm. The presence of peaks at 264 and 270 nm for AAMU-HRP and 1X-HRP conjugates, respectively, are indicative that the couplings proceeded as expected.

After the above measurements, 5  $\mu$ L of a 4 % thiomersal  
15 solution is added per mL of the AAMU-HRP or 1X-HRP conjugate solution. The conjugates are stored at 4°C.

#### Antibody production

Four mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) are used for  
20 antibody production. The protocol employed in this study was approved by the McGill University Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. Antibodies of the present invention may be monoclonal or polyclonal  
25 antibodies.

An isotonic saline solution (0.6 mL) containing 240 mg of BSA conjugated antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. A 0.5 mL aliquot of the emulsion (100 mg of antigen) is injected per rabbit

intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50 mg of antigen emulsified in incomplete Freund's adjuvant. Blood is collected by venipuncture of the ear 10-14 days after boosting. Antisera are stored at 4°C in the presence of 0.01% sodium azide.

#### Double immunodiffusion in agar plate

An 0.8% agar gel in PBS is prepared in a 60 x 15 mm petri dish. Rabbit serum albumin (100  $\mu$ L of 1 mg mL<sup>-1</sup>) conjugated to AAMU (or 1X) are added to the center well, and 100  $\mu$ L of rabbit antiserum are added to the peripheral wells. The immunodiffusion is carried out in a humidified chamber at 37°C overnight and the gel is inspected visually.

#### Antiserum titers

The wells of a microtiter plate are coated with 10  $\mu$ g mL<sup>-1</sup> of rabbit serum albumin-AAMU (or 1X) conjugate in sodium carbonate buffer, pH 9.6) for 1 hour at 37°C (100  $\mu$ L/per well). The wells are then washed three times with 100  $\mu$ L TPBS (phosphate buffer saline containing 0.05% Tween<sup>TM</sup> 20) and unoccupied sites are blocked by an incubation with 100 mL of TPBS containing 0.05% gelatin for 1 hour at 37°C. The wells are washed three times with 100  $\mu$ L TPBS and 100  $\mu$ L of antiserum diluted in TPBS are added. After 1 hour at 37°C, the wells are washed three times with TPBS, and 100  $\mu$ L of goat anti-rabbit IgGs-alkaline phosphatase conjugate, diluted in PBS containing 1% BSA, are added.

After 1 hour at 37°C, the wells are washed three times with TPBS and three times with water. To the wells are added 100 µL of a solution containing MgCl<sub>2</sub> (0.5 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min. at room temperature, the absorbency is read at 405 nm with a microplate reader. The antibody titer is defined as the dilution required to change the absorbance by one unit (1 au).

#### 10 Isolation of rabbit IgGs

The DE52-cellulose resin is washed three times with sodium phosphate buffer (500 mM, pH 7.50), the fines are removed and the resin is equilibrated with a sodium phosphate buffer (10 mM, pH 7.50). The resin is packed in a 50 x 1.6 cm column and eluted with 200-300 mL equilibrating buffer before use. To antiserum obtained from 50 mL of blood (30-32 mL) is added dropwise 25-27 mL of a 100% saturated ammonium sulfate solution with a Pasteur pipette. The suspension is left at room temperature for 3 h and centrifuged for 30 min. at 2560 g at 20°C. The pellet is dissolved with 15 mL sodium phosphate buffer (10 mM, pH 7.50) and dialyzed at room temperature with the buffer changed twice per day. The dialyzed solution is centrifuged at 2560 g for 10 min. at 20°C to remove precipitate formed during dialysis. The supernatant is applied to the ion-exchange column. Fractions of 7 mL are collected. After application, the column is eluted with the equilibrating buffer until the absorbance at 280 nm becomes less than 0.05 au. The column is then eluted with the equilibrating buffer containing 50

mM NaCl. Fractions having absorbencies greater than 0.2 at 280 nm are saved and stored at 4°C. Protein concentrations of the fractions are determined as described above.

5 Competitive antigen ELISA

Buffers and water without additives are filtered through millipore filters and kept for 1 week. BSA, antibodies, Tween<sup>TM</sup> 20 and horse radish peroxidase conjugates are added to these buffers and water just prior to use. Urine samples are usually collected 4 hours after drinking a cup of coffee (instant or brewed with approximately 100 mg of caffeine per cup) and stored at -80°C. The urine samples are diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and are subsequently diluted with water to give concentrations of AAMU and 1X no higher than  $3 \times 10^{-6}$  M in the ELISA. All the pipettings are done with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing 2.5 µg mL<sup>-1</sup> antibodies are added to each well. After 90 min. at room temperature, the wells are washed three times with 100 mL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% Tween<sup>TM</sup> 20.

After the initial wash, unoccupied sites are blocked by incubation for 90 min. at room temperature with 100 µL TBP containing 3% BSA. The wells are washed four times with 100 µL TPB. The washing is followed by additions of 50 µL of 12 mg mL<sup>-1</sup> AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50 µL of either water, standard (13

standards; AAMU or 1X,  $2 \times 10^{-4}$  to  $2 \times 10^{-8}$  M) or sample in duplicate. The microplate is gently shaken with an orbital shaker at room temperature for 3-4 hours. The wells are washed three times with 100  $\mu$ L TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate is added 150  $\mu$ L of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction is stopped with 50  $\mu$ L of 2.5 M HCl. After shaking the plate 3 min., the absorbances are read with a microtiter plate reader at 490 nm.

## Results

Polyclonal antibodies against AAMU and 1X could be successfully raised in rabbits after their conjugation to bovine serum albumin. Each rabbit produced antibody titers of 30,000-100,000 as determined by ELISA. This was also indicated by strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin. On this basis, a) IgGs antibodies were isolated on a DE-52 cellulose column and b) a competitive antigen ELISA for NAT2 phenotyping using caffeine as probe substrate was developed according to the methods described in the above section entitled Materials and Methods.

Contrary to current methods used for phenotyping, the assay involves no extraction, is sensitive and rapid, and can be readily carried out on a routine basis by a

technician with a minimum of training in a clinical laboratory.

The present invention will be more readily understood by referring to the following examples which are given to  
5 illustrate the invention rather than to limit its scope.

A competitive antigen ELISA for NAT2 phenotyping using caffeine as a probe substrate

Buffers and water without additives were filtered  
10 through millipore filters and kept for 1 week. BSA, antibodies, Tween™ 20 and horse radish peroxidase conjugates were added to these buffers and water just prior to use. Urine samples were usually collected 4 hours after drinking a cup of coffee (instant or brewed with  
15 approximately 100 mg of caffeine per cup) and stored at - 80°C. They were diluted 10 times with sodium phosphate buffer (620 mosm, pH 7.50) and were subsequently diluted with water to give concentrations of AAMU and 1X no higher than  $3 \times 10^{-6}$  M in the ELISA. All the pipettings were done  
20 with an eight-channel pipette, except those of the antibody and sample solutions. Starting with the last well, 100 µL of a carbonate buffer (100 mM, pH 9.6) containing  $2.5 \mu\text{g mL}^{-1}$  antibodies was pipetted. After 90 min. at room temperature, the wells were washed three  
25 times with 100 µL of TPB: isotonic sodium phosphate buffer (310 mosm, pH 7.50) containing 0.05% Tween™ 20.

After the initial wash, unoccupied sites were blocked by incubation for 90 min. at room temperature with 100 µL TBP containing 3% BSA. The wells were washed four times

with 100  $\mu$ L TPB. This was followed by additions of 50  $\mu$ L of 12 mg mL<sup>-1</sup> AAMU-HRP or 1X-HRP conjugate in 2 x TPB containing 2% BSA, and 50  $\mu$ L of either water, standard (13 standards; AAMU or 1X,  $2 \times 10^{-4}$  to  $2 \times 10^{-8}$  M) or sample in duplicate. The microplate was gently shaken with an orbital shaker at room temperature for 3-4 hours. The wells were washed three times with 100  $\mu$ L with TPB containing 1% BSA and three times with water containing 0.05% Tween™ 20. To the washed plate was added 150  $\mu$ L of a substrate buffer composed of citric acid (25 mM) and sodium phosphate dibasic buffer (50 mM, pH 5.0) containing 0.06% hydrogen peroxide and 0.04% o-phenylenediamine hydrochloride. After 20 min. at room temperature with shaking, the reaction was stopped with 50  $\mu$ L of 2.5 N HCl. After shaking the plate 3 min., the absorbances were read with a microtiter plate reader at 490 nm.

The competitive antigen ELISA curves of AAMU-Ab and 1X-Ab determinations obtained in duplicate are presented in Fig. 18. Each calibration curve represents the average of two calibration curves. The height of the bars measure the deviations of the absorbency values between the two calibration curves. Data points without bars indicate that deviations of the absorbency values are equal or less than the size of the symbols representing the data points.

Under the experimental conditions of the ELISA: background was less than 0.10 au; the practical limits of detection of AAMU and 1X were  $2 \times 10^{-7}$  M and  $2 \times 10^{-6}$  M, respectively, concentrations 500 and 50 times lower than those in urine samples from previous phenotyping studies

(Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, 47:470-477); the intra-assay and interassay coefficients of variations of AAMU and 1X were 15-20% over the concentration range of 0.01-0.05 mM.

5        A variety of conditions for the ELISA were tested and a number of noteworthy observations were made: gelatin, which was used in the competitive antigen ELISA determination of caffeine in plasma (Fickling, S.A. et al. (1990) *J. Immunol. Meth.*, 129:159-164), could not be used  
10    in our ELISA owing to excessive background absorbency which varied between 0.5 and 1.0 au; in the absence of Tween™ 20, absorbency changes per 15 min. decreased by a factor of at least 3, and calibration curves were generally erratic; absorbency coefficients of variation of  
15    samples increased by a factor of 3 to 4 when the conjugates and haptens were added to the wells as a mixture instead individually.

      The cross reactivities of AAMU-Ab and 1X-Ab were tested using a wide variety of caffeine metabolites and  
20    structural analogs (Table 5 below). AAMU-Ab appeared highly specific for binding AAMU, while 1X-Ab appeared relatively specific for binding 1X. However, a 11% cross reactivity was observed with 1-methyluric acid (1U), a major caffeine metabolite.



Table 5

**Cross-reactivity of AAMU-Ab and 1X-Ab towards different caffeine metabolites and structural analogs**

Compound	% Cross-Reaction	
	AAMU-Ab	1X-Ab
Xanthine	0 <sup>a</sup>	0
Hypoxanthine	0	0
1-Methyl Xanthine (1X)	0	100
3-Methyl Xanthine	0	0
7-Methyl Xanthine	0	0
8-Methyl Xanthine	0	0
1,3-Dimethyl Xanthine (Theophylline)	0	0.2
1,7-Dimethyl Xanthine (Paraxanthine)	0	0.5
3,7-Dimethyl Xanthine (Theobromine)	0	0
1,3,7-Trimethyl Xanthine (Caffeine)	0	0
Uric acid	0	0
1-Methyluric acid	0	11
1,7-Dimethyluric acid	0	0
Guanine	0	0
Uracil	0	0
5-Acetamino-6-amino-uracil	0.6	0
5-Acetamino-6-amino-1-methyluracil (AAMU)	100	0
5-Acetamino-6-amino-1,3-dimethyluracil	0	0

a. The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest compound concentration tested in the ELISA ( $5 \times 10^{-3}$  M); concentrations of 5-acetamino-6-amino-1-methyluracil (AAMU) and 1-Methyl Xanthine (1X) required for 50% inhibition in the competitive antigen ELISA were  $1.5 \times 10^{-6}$  M and  $10^{-5}$  M, respectively.

The relative high level of cross reactivity of 1U is, however, unlikely to interfere significantly in the determination of 1X and the assignment of NAT2 phenotypes, since the ratio of 1U:1X is no greater than 2.5:1 in 97%

of the population (Tang, B-K. et al. (1991) *Clin. Pharmacol. Ther.*, 49:648-657). This is confirmed by measurements of apparent concentrations of 1X when the ratio varied between 0-8.0 at the fixed 1X concentration of  $3 \times 10^{-6}$  M (Table 6 below). At 1U:1X ratios of 2.5 and 3.0, the apparent increases were 22% and 32%, respectively.

**Table 6**

**The effect of the ratio 1U:1X on the determination of 1X concentration by ELISA at fixed 1X concentration of  $3 \times 10^{-6}$  M**

1U:1X ratio	[1X] x $10^6$ (M)
0.0	3.00
0.50	2.75
1.00	3.25
1.50	3.25
2.00	3.60
2.50	3.65
3.00	3.95
4.00	4.20
5.00	4.30
6.00	4.50
8.00	4.30

The following observations attested to the validity of the competitive antigen ELISA for NAT2 phenotyping.

1) The ELISA assigned the correct phenotype in 29 of 30 individuals that have been phenotyped by capillary electrophoresis (CE) (Lloyd, D. et al. (1992) *J. Chrom.*, 578:283-291).

2) In the CE method, the phenotype was determined using AFMU/1X peak height ratios rather than the AAMU/1X molar ratios used in the ELISA. When the molar ratios determined by ELISA and the peak height ratios determined

by CE were correlated by regression analysis, the calculated regression equation was  $y = 0.48 + 0.87 x$ , with a correlation coefficient <sup>®</sup> of 0.84, Taking account that these two ratios are not exactly equal and that Kalow and Tang (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, 53:503-514) have pointed out that using AFMU rather than AAMU can lead to misclassification of NAT2 phenotypes, there is a remarkable agreement between the two methods.

3) The ELISA was used in determining the NAT2 phenotype distribution within a group of 146 individuals. Fig. 19 illustrates a histogram of the NAT2 phenotypes of this group as determined by measuring the AAMU/1X ratio in urine samples by ELISA. Assuming an antimode of 1.80, the test population contained 60.4% slow acetylators and 39.6% fast acetylators. This is consistent with previously reported distributions (Kalow, W. et al. (1993) *Clin. Pharmacol. Ther.*, 53:503-514; Kilbane, A.J. et al. (1990) *Clin. Pharmacol. Ther.*, 47:470-477).

Determination of 5-acetamino-6-amino-1-methyluracil (AAMU)  
and 1-methyl xanthine in urine samples  
with the ELISA kit

5 **Table 7**  
**Content of the ELISA kit and conditions of storage**

	Item Unit	State	Amt	Storage conditions	
10	Tween™ 20	1 vial	Liquid	250 µL/vial	4°C
	H <sub>2</sub> O <sub>2</sub>	1 vial	Liquid	250 µL/vial	4°C
15	AAMU-HRP	1 vial	Liquid	250 µL/vial	4°C
	1X-HRP	1 vial	Liquid	250 µL/vial	4°C
	Buffer A	4 vials	Solid	0.8894 g/vial	4°C
20	Buffer B	6 vials	Solid	1.234 g/vial	4°C
	Buffer C	6 vials	Solid	1.1170 g/vial	4°C
	Buffer D	6 vials	Solid	0.8082 g/vial	4°C
25	Plate(AAMU-Ab)	2	Solid	-	4°C
	Plate (1X-Ab)	2	Solid	-	4°C
30	Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
	Standards (AAMU)	14 vials	Liquid	200 µL	-20°C
	Standards(1X)	14 vials	Liquid	200 µL	-20°C
35	1N NaOH	1 bottle	Liquid	15 mL	20°C
	1N HCl	1 bottle	Liquid	15 mL	20°C

## Conversion of AFMU to AAMU

In order to determine the AAMU concentrations in urine samples by competitive antigen ELISA, a transformation of AFMU to AAMU is required.

- 5     • Thaw and warm up to room temperature the urine sample.
- Suspend the sample thoroughly with the vortex before pipeting.
- Add 100  $\mu$ L of a urine sample to a 1.5 mL-microtube.
- Add 100  $\mu$ L of a 1N NaOH solution.
- 10    • Leave at room temperature for 10 min.
- Neutralize with 100  $\mu$ L 1N HCl solution.
- Add 700  $\mu$ L of Buffer A (dissolve the powder of one vial A/50 mL).

- 15    Dilutions of urine samples for the determinations of [AAMU] and [1X] by ELISA

The dilutions of urine samples required for determinations of AAMU and 1X are a function of the sensitivity of the competitive antigen ELISA and AAMU and 1X concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X concentrations are about  $3 \times 10^{-6}$  M in the well of the microtiter plate. Generally, dilution factors of 100-400 and 50-100 have been used for AAMU and 1X, respectively.

**Table 8****Microtube #**

	20x	40x	50x	80x	100x	150x	200x	400x
5 Dilution Factor								
Solution	1	2	3	4	5	6	7	8
Urine sample(mL) <sup>a</sup>	500	250	200	125	100	66.7	50	25
10 10 x diluted Buffer B (mL)	500	750	800	875	900	933.3	950	975

a. Vortex the microtubes containing the urine sample before pipeting.

Store the diluted urine samples at -20°C.

Buffer B: dissolve the content of one vial B/100 mL

### 15 Determination of [AAMU] and [1 X] in diluted urine samples by ELISA

#### Precautions

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (Substrate buffer). Each sample is  
 20 determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbance values of duplicates should be within less than 5%. Buffers C, D and E are freshly prepared. Buffer E-H<sub>2</sub>O<sub>2</sub> is prepared just prior pipeting in the microtiter plate  
 25 wells.

#### Preparation of samples:

Prepare Table 9 with a computer and print it. This table shows the content of each well of a 96-well  
 30 microtiter plate. Enter the name of the urine sample (or number) at the corresponding well positions in Table 9. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 9. Enter the dilution of each urine sample with buffer B at the

- corresponding position in Table 9: for example, for a D.F. of 100 (100  $\mu$ L of 10x diluted urine sample + 900  $\mu$ L buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the
- 5 different dilutions. Prepare the different dilutions of the urine samples in 1.5-mL microtubes. Prepare Table 10 with a computer and print it. Prepare the following 48 microtubes in the order indicated in Table 10.

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**Table 9**  
**Positions of blanks, control and urine samples in a microtiter plate**

Sample	Well #	D.F	Dil.	Sample	Well #	D.F	Dil.
Blank	1-2	-		Control	49-50	-	
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72	-	
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96	-	

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**Table 10**  
**Content of the different microtubes**

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	AAMU or 1X	27	9	Dil. Urine
4	S2	AAMU or 1X	28	10	Dil. Urine
5	S3	AAMU or 1X	29	11	Dil. Urine
6	S4	AAMU or 1X	30	12	Dil. Urine
7	S5	AAMU or 1X	31	13	Dil. Urine
8	S6	AAMU or 1X	32	14	Dil. Urine
9	S7	AAMU or 1X	33	15	Dil. Urine
10	S8	AAMU or 1X	34	16	Dil. Urine
11	S9	AAMU or 1X	35	17	Dil. Urine
12	S10	AAMU or 1X	36	Control	Buffer B
13	S11	AAMU or 1X	37	18	Dil. Urine
14	S12	AAMU or 1X	38	19	Dil. Urine
15	S13	AAMU or 1X	39	20	Dil. Urine
16	S14	AAMU or 1X	40	21	Dil. Urine
17	S15	AAMU or 1X	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

**Solutions:**

- 5 Buffer C: Dissolve the content of one vial C/50 mL water.  
 Add 25 mL of Tween™ 20.

Buffer D: Dissolve the content of one vial D /25 mL water.  
 Add 25 mL of Tween™ 20.

0.05 % Tween™ 20: Add 25 µL of Tween™ 20 to a 100-mL erlenmeyer flask containing 50 mL of water.

2.5 N HCl: 41.75 mL of 12 N HCl/200 mL water.  
5 Store in a 250-mL glass bottle.

AAMU-HRP conjugate: Add 9 mL of Buffer C to a 15-mL glass test tube. Add 90 µL of AAMU-HRP stock solution.

10 1X-HRP conjugate: Add 9 mL of a??? Which buffer? 2 % BSA solution to a 15-mL glass test tube.  
Add 90 µL 1X-HRP stock solution.

15 Buffer E-H<sub>2</sub>O<sub>2</sub>: Dissolve the content of one vial E-substrate/50 ml water. Add 25 µL of a 30 % H<sub>2</sub>O<sub>2</sub> solution (prepared just prior to adding to the microtiter plate wells).

**Table 11**  
**Standard solutions of AAMU and 1X**  
**(diluted with buffer B)**

	AAMU		1 X	
	Standard	[AAMU]	Standard	[1X]
10	1	$1.12 \times 10^{-4}$ M	1	$2.00 \times 10^{-4}$ M
	2	$6.00 \times 10^{-5}$ M	2	$1.12 \times 10^{-4}$ M
	3	$3.56 \times 10^{-5}$ M	3	$6.00 \times 10^{-5}$ M
	4	$2.00 \times 10^{-5}$ M	4	$3.56 \times 10^{-5}$ M
	5	$6.00 \times 10^{-6}$ M	5	$2.00 \times 10^{-5}$ M
15	6	$3.56 \times 10^{-6}$ M	6	$1.12 \times 10^{-5}$ M
	7	$2.00 \times 10^{-6}$ M	7	$6.00 \times 10^{-6}$ M
	8	$1.12 \times 10^{-6}$ M	8	$3.56 \times 10^{-6}$ M
	9	$6.00 \times 10^{-7}$ M	9	$2.00 \times 10^{-6}$ M
	10	$3.56 \times 10^{-7}$ M	10	$1.12 \times 10^{-6}$ M
20	11	$2.00 \times 10^{-7}$ M	11	$6.00 \times 10^{-7}$ M
	12	$1.12 \times 10^{-7}$ M	12	$3.56 \times 10^{-7}$ M
	13	$6.00 \times 10^{-8}$ M	13	$2.00 \times 10^{-7}$ M
	14	$3.56 \times 10^{-8}$ M	14	$1.12 \times 10^{-7}$ M
	15	$2.00 \times 10^{-8}$ M	15	$6.00 \times 10^{-8}$ M

**25 Conditions of the ELISA**

Add 50  $\mu$ L/well of AAMU-HRP (or 1X-HRP) conjugate solution, starting from the last row. Add 50  $\mu$ L/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200  $\mu$ L), starting from well # 96 (see Table 9). Cover the plate and mix gently by vortexing for several seconds. Leave the plate at room temperature for 3 h. Wash 3 times with 100  $\mu$ L/well with buffer C, using a microtiter plate washer. Wash 3 times with 100  $\mu$ L/well with the 0.05% Tween™ 20 solution. Add 150  $\mu$ L/well of Buffer E-

H<sub>2</sub>O<sub>2</sub> (prepared just prior adding to the microtiter plate wells). Shake 20-30 min at room temperature with an orbital shaker. Add 50 µL/well of a 2.5 N HCl solution. Shake 3 min with the orbital shaker at room temperature.

- 5 Read the absorbance of the wells with microtiter plate reader at 490 nm. Print the sheet of data and properly identify the data sheet.

- 10 Calculation of the [AAMU] and [1X] in urine samples from the data

- Draw a Table 12 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 12. Draw the calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknown from the calibration curve and enter the data in Table 13. Multiply the [AAMU] (or [1X]) of the unknown by the dilution factor and enter the result in the corresponding case of Table 13.

**Table 12****Average absorbance values of samples in the microtiter plate**

5

Sample	Well #	A <sub>490</sub>	Sample	Well #	A <sub>490</sub>
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 13  
AAMU (or 1X) concentrations in urine samples

Sample	D.F.	[AAMU]	[AAMU] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
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**Table 14**  
**Composition of the different buffer**

Buffer	pH	Composition	(mM)	Concen. (mM)	[P]
5					
A	7.50	0.15629 g/100 mL NaH <sub>2</sub> PO <sub>4</sub>		11.325	
		1.622 g/100 mL Na <sub>2</sub> HPO <sub>4</sub> ·7 H <sub>2</sub> O		60.099	
		1.778 g/100 mL (total weight)			71.424
10					
B	7.50	0.1210191 g /100 mL NaH <sub>2</sub> PO <sub>4</sub>		8.769	
		1.11309 g /100 mL of Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O		41.23	
		1.2341 g/100 mL (total weight)			49.999
15					
C	7.50	1 g/100 mL of BSA		-	
		0.1210191 g /100 mL of NaH <sub>2</sub> PO <sub>4</sub>		8.769	
		1.11309 g /100 mL of Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O		41.23	
		2.2341 g/100 mL (total weight)			49.999
20					
D	7.50	2 g/100 mL of BSA			
		0.1210191 g /100 mL of NaH <sub>2</sub> PO <sub>4</sub>		8.769	
		1.11309 g /100 mL of Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O		41.23	
		3.2341 g/100 mL (total weight)			49.999
25					
E	5.00	0.52508 g/100 mL of citric acid		25	
		1.34848 g/100 mL of Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O		50	
		40 mg/100 mL of o-phenylenediamine hydrochloride			
		1.913567 g/100 mL (total weight)			-
30					

### CYP1A2

Different probe substrates can be used to determine the CYP1A2 phenotype (caffeine, theophylline) In accordance with the present invention suitable probe substrates include without limitation, caffeine, theophylline or acetaminophen.

Of these caffeine is the preferred probe substrate. Caffeine is widely consumed and relatively safe. In previous studies the phenotype has been generally determined from the ratios of 1,7-dimethylxanthine (1,7 DMX) + 1,7-dimethyluric acid (1,7 DMU) and 1,3,7-

trimethylxanthine (1,3,7 TMX, caffeine). In these studies, the subjects are given an oral dose of a caffeine containing-substance, and the urinary concentrations of the target metabolites determined by HPLC (Kilbane, A. J. et al. (1990) Clin. Pharmacol. Ther 47: 470-477; Tang, B.-K. et al. (1991) Clin. Pharmacol. Ther 49: 648-657) or CE (Meachers et al. (1998) Biomarkers 3: 205-218).

Inhibition of CYP1A2 by quinolone antibiotic agents or serotonin reuptake inhibitors, may result in theophylline toxicity. For these reasons, the utility of a reliable phenotyping test is evident.

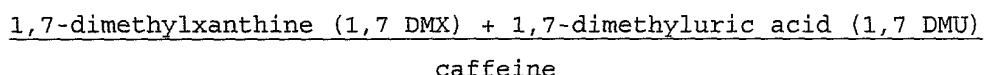
Enzyme linked immunosorbent assays (ELISA) have been successfully applied in the determination of low amounts of drugs and other antigenic compounds in plasma and urine samples and are simple to carry out. We have previously developed an ELISA for N-acetyltransferase-2 (NAT2) phenotyping using caffeine as a probe substrate (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) J. Pharm. Biomed. Anal. 13: 1079-1086). We have subsequently tested and proven the validity of the ELISA for the NAT2 phenotyping (Leyland-Jones et al. (1999) Amer. Assoc. Cancer Res. 40: Abstract 356). The ELISA for NAT2 phenotyping is simpler to carry out than the HPLC and CE.

In accordance with the present invention, there are currently being developed antibodies to measure the molar ratio of caffeine and two caffeine metabolites (1,7-dimethylxanthine (1,7 DMX) and 1,7-dimethyluric acid (1,7 DMU)) in urine samples of an individual collected after caffeine consumption. This ratio provides a determination of an individual's CYP1A2 phenotype. Subsequently, there



will be an antigen enzyme linked immunosorbent assay (ELISA) for measuring this ratio using these antibodies. The antibodies of the present invention can be polyclonal or monoclonal antibodies raised against caffeine and two  
5 different metabolites of caffeine, which allow the measurement of the molar ratio of caffeine and these metabolites.

In accordance with the present invention, the molar ratio of caffeine metabolites is used to determine the  
10 CYP1A2 phenotype of the individual as follows:



Molar ratios of 4 and 12 separate slow, intermediate  
15 and fast CYP1A2 metabolizers (Butler et al. (1992) Pharmacogenetics 2: 116-117).

## MATERIALS AND METHODS

### Materials

20 N-acetyl-p-aminophenol (acetaminophen), dioxane, formic acid 98-100 % glass redistilled and isobutyl chloroformate are purchased from A&C American Chemicals Ltd. (Ville St-Laurent, Que. Canada); horse radish peroxidase is purchased from Boehringer Mannheim (Montreal, Que.,  
25 Canada); ELISA plates (96-well Easy Wash™ modified flat bottom, high binding; Corning glass wares, Corning, NY, USA) and Falcon 96-well microtest tissue culture plate, no. 3072 (Beckton Dickinson Labware, Franklin, NJ, USA) are purchased from Fisher (Montreal, Quebec, Canada);  
30 alkaline phosphatase conjugated to goat anti-rabbit IgGs, Keyhole limpet hemocyanin (KLH) is from Pierce Chemical

Co. (Rockford, IL, USA); acetic anhydride, acetonitrile  
HPLC grade, benzylurea, bovine serum albumin (Cat. No A-  
3803), N-bromosuccinimide, caffeine metabolites; 1-ethyl-  
3-(3-dimethylaminopropyl) carbodiimide hydrochloride  
5 solution (EDAC), ethyl 4-bromobutyrate, ethyl 6-bro-  
mohexanoate, methyl cyanoacetate, deuterated chloroform  
(CDCl<sub>3</sub>) , deuterated dimethylsulfoxide (d<sub>6</sub>), deuterated  
oxide (D<sub>2</sub>O), 1,4-diaminobutane, diethanolamine, dimethyl-  
formamide, dimethylsulfate, di-tert-butyl dicarbonate,  
10 ethyl chloroformate, Freund's adjuvant (complete and  
incomplete), glutaraldehyde (50 % v/v), 1-methylxanthine,  
p-nitrophenolphosphate disodium salt, palladium, 10 wt. %  
(dry basis) on activated carbon, o-phenylenediamine  
hydrochloride, polyoxyethylene sorbitan monolaurate  
15 (Tween<sup>TM</sup> 20), porcine skin gelatin, protein A-Sepharose 4B,  
Sephadex<sup>TM</sup> G25 fine, sodium hydride, sodium methoxide,  
theophylline, tributylamine, Tween<sup>TM</sup> 20, are purchased from  
Sigma-Aldrich (St-Louis, Missouri, USA); Silica gel  
particle size 0.040-0.063 mm (230-400 mesh) ASTM Emerck  
20 Darmstadt, Germany is purchased from VWR (Montreal, Que.,  
Canada). Dioxane is dried by refluxing over calcium  
hydride for 4 hours and distilled before use. Other  
reagents are ACS grade.

## 25 Synthetic procedures

The synthetic routes for the production of caffeine,  
1,7-dimethylxanthine, 1,7-dimethyluric acid derivatives  
are shown in Figs. 20 and 21.

Synthesis of 7-ethoxycarboxypentyl-1,3-dimethylxanthine (II)

Compound II is synthesized by a procedure similar to that of Daly et al. (Daly, J.W., Mueller, C., Shamin, M. (1991) Pharmacology, 42: 309-321). First, 320 mg of theophylline (I) (1.78 mmole) is dissolved in 7 mL of dry dimethylformamide and then 290 mg of potassium carbonate (2.1 mmole) is added to the reaction mixture. Then, 358  $\mu$ L of ethyl 6-bromohexanoate (2.02 mmole) is slowly added and the suspension is heated at 60°C for 14 hours. The suspension is filtered in order to remove the potassium carbonate. After washing the potassium carbonate with some dimethylformamide, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in chloroform and the solution is dried over magnesium sulfate ( $\text{MgSO}_4$ ). The solvent is evaporated under reduced pressure with a rotary evaporator. 480 mg of the product (slightly yellow oil 1.49 mmole) is obtained, corresponding to a yield of 83.7%.

Synthesis of 7-carboxypentyl-1,3-dimethylxanthine (III)

Compound III is synthesized as follows. First, 225 mg of compound II (0.7 mmole) is dissolved in 7 mL of dimethylformamide. Then, 4 mL of a 10% NaOH solution is added and the solution is refluxed for 30 min (100-125 °C). The solvents are evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in 7 mL of water and the solution is acidified to pH 4 with a 6N HCl solution. Cooling the solution at 4°C crystallizes the product as needle-like crystals. The crystals are filtered under vacuum through a 15-mL sin-

tered glass funnel (10-15 ASTM) and dried. A total of 175 mg of the product is obtained (0.595 mmole), corresponding to a yield of 85%.

5 Synthesis of 7-methoxycarboxypentyl-1-methylxanthine (V)

Compound V is synthesized as follows. First, 116 mg of 1-methylxanthine (IV) (0.7 mmole) is dissolved in 4 mL of dimethylformamide. Then, 129 mg of potassium carbonate (0.93 mmole) is added and the resulting solution is  
10 stirred. Then, 125  $\mu$ L of ethyl-6-bromohexanoate (0.7 mmole) in 0.4 mL dimethylformamide is slowly added in three portions. The reaction mixture is heated at 50  $^{\circ}$ C for 1.5 hours and at 65  $^{\circ}$ C for 1 hour. After cooling, the suspension is filtered and the filtrate is evaporated  
15 under reduced pressure with a rotary evaporator and a high vacuum pump. The product is purified by flash chromatography on a silica gel column (40 x 1 cm) using an ethyl acetate-hexane solution (9:1, v/v) as the eluent.

20 Synthesis of 7-carboxypentyl-1-methylxanthine (VI)

Compound VI is synthesized as follows. First, 31 mg of compound V (0.1 mmol) is dissolved in 1 mL of dimethylformamide and then 660  $\mu$ L of 10% NaOH is added. The resulting solution is refluxed for 30 min (100-120  $^{\circ}$ C). After  
25 cooling at room temperature, the solvent is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The residue is dissolved in water and acidified to pH 4 with a 6N HCl solution. Upon cooling, the solution yields white needle-like crystals, which are

filtered and dried. A total of 23 mg of the product (0.082 mmole) is obtained, corresponding to a yield of 82%.

#### Synthesis of 6-amino-1-benzyl uracil (IX)

5       Compound IX is synthesized according to the procedure similar of that of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. First, 8.64 g of sodium methoxide (160 mmol) is dissolved in 71 mL methanol. The solution is  
10       stirred and 7.55 g of benzylurea (50 mmol) and 4.71 mL methyl cyanoacetate (53.4 mmol) are added. The suspension is refluxed 5.5 hours at 68-70°C and cooled at room temperature. After filtration, the methanol is evaporated under reduced pressure with a rotary evaporator. The  
15       residue is dissolved in warm distilled water, and the product is precipitated by acidification to pH 3-4 with glacial acetic acid. After 2 hours (or overnight) at room temperature, the suspension is filtered under vacuum through a sintered glass funnel. The product is washed  
20       with water and dried. The yield is 62-65%.

#### Synthesis of 6-amino-1-benzyl-5-bromouracil (X)

      Compound X is synthesized according to the procedure of Hutzenlaub and Pfeiderer (Hutzenlaub, W., and  
25       Pfeiderer, W. (1979). Liebigs Ann. Chem. 1847-1854) as follows. First, 3.2 g of 6-amino-1-benzyluracil (15.8 mmol) is dissolved at 100° C in 60 mL acetic acid and 3 mL acetic anhydride. Then, 2.85 g of N-bromosuccinimide (16 mmol) is added in small portions over the next 30 minutes.  
30       The reaction mixture is stirred for 1 hour and cooled at

room temperature. The precipitate is filtered and washed with small amounts of cold ethanol and dried. A total of 3.36 g of white crystals are obtained (12 mmol), corresponding to a yield of 76%.

5

Synthesis of 6-amino-1-benzyl-5-[N-4'-aminobutyl]-amino]uracil (XI)

Compound XI is synthesized as follows. First, 3 g of compound X (10.71 mmol) is dissolved in 30 mL of 50% 1,4-diaminobutane (bp 158-160°; d 0.877) in water (v/v) and the solution is stirred overnight at room temperature. The solution is evaporated under reduced pressure with a rotary evaporator and a high vacuum pump. The resulting oil is dissolved in a minimal amount of ethyl acetate-methanol solution (4:1; v/v) and is purified by dry flash chromatography on a silica gel packed in a sintered glass funnel (150 mL) with ethyl acetate-methanol solutions as the eluents. At each successive fraction, the solvent polarity is increased, varying from 60% ethylacetate/40% methanol to 45% ethylacetate/55% methanol (v/v). The product is isolated as a light yellow oil. The amount of purified product obtained is 1.69 g (6.1 mmol), corresponding to a yield of 57%.

25 Synthesis of 6-amino-1-benzyl-5-[N-4'-tert-butoxycarbonyl-amino]uracil (XII)

Compound XII is synthesized as follows. First, 1.63 g of compound XI (5.9 mmol) is dissolved in 5.4 mL of a 1 N NaOH solution. Then, 270 mg of sodium bicarbonate (3.2 mmol) and 2.7 mL of water are added. Then, 5.4 mL of di-tert-butyl dicarbonate solution in isopropanol (1.88 g

(8.61 mmol) is dissolved in 5.4 mL isopropanol) is added slowly to the solution of compound XI. After stirring for 3 hours at room temperature, 13.4 mL of water is added and the unreacted di-tert-butyl dicarbonate is extracted twice with 20 mL of petroleum ether. The pH of the reaction mixture is adjusted to 7 by the addition of a 10% citric acid solution and the solution is extracted twice with 40 mL ethyl acetate. The organic layer is dried over sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) and is concentrated under reduced pressure with a rotary evaporator. The product is precipitated by the addition of some light petroleum ether to the concentrated solution. The amount of product obtained is 0.99 g of an off-white crystalline compound XII (2.62 mmol), corresponding to a yield of 44%.

Synthesis of 6-amino -1- benzyl-5-[(N-4'tert-butoxy-carbonylaminobutyl-N-ethoxycarbonyl)-amino]-uracil (XIII)

Compound XIII is synthesized as follows. First, 806 mg of compound XII (2.14 mmol) is suspended in 7.5 mL of water and stirred energetically. Then, 0.5 mL of ethyl chloroformate (5.22 mmol) is added. Then, 3.75 mL of a 1N NaOH solution is added dropwise and the resulting solution is stirred at room temperature for 2.5 hours. The white solid product is filtered, washed thoroughly with water and dried. A total of 741 mg of the product is obtained (1.77 mmol), corresponding to a yield of 82.7%.

Synthesis of 6-amino-1-benzyl-5-[(N-4'-tert-butoxy carbonylaminobutyl-N-ethoxycarbonyl)-amino]-3-methyl uracil (XIV)

Compound XIV is synthesized as follows. First, 712 mg  
5 of compound XIII (1.77 mmol) is suspended in 5.8 mL of  
water. Then, 2.3 mL of a 1N NaOH solution are added and  
the resulting solution is heated at 40° C and vigorously  
stirred. Then, 0.23 mL dimethylsulfate (2.43 mmol) is  
slowly added and the resulting solution stirred at 40 ° C  
10 for 1.5 hours. The precipitate, which forms during the  
reaction, is filtered, washed with water and dried. The  
product is purified from the precipitate by flash  
chromatography on a silica gel column (40 x 1cm) using a  
solution of 4% methanol in dichloromethane as eluent. The  
15 product is recrystallized from ethyl acetate. A total of  
498 mg of compound XIV (1.15 mmol) is obtained,  
corresponding to a yield of 65%.

Synthesis of 6-amino-5-[(N-4'-tert-butoxycarbonyl-amino-  
20 butyl-N-ethoxycarbonyl)-amino]-3-methyluracil (XV)

Compound XV is synthesized as follows. First, 440 mg  
of compound XIV (1.02 mmol) is dissolved in 12 mL methanol  
and mixed with 252 mg ammonium formate (4 mmol). Then, 240  
25 mg of palladium-on-charcoal (10%) are added under nitrogen  
atmosphere. The catalytic hydrogenation is performed at  
room temperature for 3 hours. The catalyst is removed by  
filtration and the filtrate is evaporated under reduced  
pressure with a rotary evaporator and a high vacuum pump.  
30 A total of 341 mg of the product is obtained (0.99 mmol),  
corresponding to a yield of 97%.



## Synthesis of 7-(4' aminobutyl)-1-methyluric acid (XVI)

Compound XVI is synthesized as follows. First, 300 mg  
5 of compound XV (0.875 mmol) is dissolved in 4.5 mL dry  
dimethylformamide and the resulting solution is mixed with  
144 mg of sodium hydride (6 mmol). The mixture is stirred  
at room temperature for 20 min and at 110-115 °C for 30  
min. The color changes slowly to a dark yellow. After  
10 cooling, 6.5 mL of water are added and the solution is  
acidified to pH 0 with a 6N HCl solution. The solvents are  
evaporated under reduced pressure with a rotary evaporator  
and a high vacuum pump, and the crude product is dissolved  
in a ethyl acetate-methanol solution (1:4, v/v). The  
15 inorganic salt is removed by filtration and the yellow  
filtrate is purified by flash chromatography on a silica  
gel column (40 x 1 cm) using a solution of ethyl acetate-  
methanol (3:7, v/v) as the eluent. The fraction containing  
the pure product is evaporated under reduced pressure with  
20 a rotary evaporator. After titration of the residue with  
isopropanol, the product is obtained as a pale yellow  
solid. A total of 98.9 mg of the product is obtained  
(0.391 mmol), corresponding to a yield of 45%.

## 25 NMR Spectroscopy

<sup>1</sup>H NMR spectra of synthesized are obtained using a 500  
mHz spectrophotometer (Varian XL 500 mHz, Varian  
Analytical Instruments, San Fernando, CA, USA).

## Coupling of haptens to horse radish peroxidase

The caffeine and 1,7-dimethylxanthine derivatives and the 1,7-dimethyluric acid derivative (after succinylation with succinic anhydride) are conjugated to horse radish peroxidase (HRP) by the following procedure. To a 5 mL round bottom flask 0.12 mmol of the derivative are added. Then, 500  $\mu$ L of dioxane freshly dried over calcium chloride are added. The suspension is stirred and cooled at 10° C in a water bath using crushed ice. Then, 31  $\mu$ L isobutylchloroformate (0.24 mmol) (recently opened or purchased) and 114  $\mu$ L tributylamine (0.47 mmol) are added. The suspension is stirred for 30 min at 10° C. While stirring, a solution is prepared by dissolving 13 mg of horse radish peroxidase (HRP) in 2 mL of water. The solution is cooled at 4° C on crushed ice. After the 30 min. stirring, 100  $\mu$ L of a 1 N NaOH solution (freshly prepared) at 4°C is added to the HRP solution and the alkaline HRP solution is poured at once into the 5 mL flask. The suspension is stirred for 4 hours at 10-12° C. The free derivative is separated from the HRP conjugate by filtration on a Sephadex G-25<sup>TM</sup> fine column (1.6 x 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.0. The fractions of 1.0-1.2 mL are collected manually or with a fraction collector. During elution two bands may be observed: the HRP conjugate and a light yellow band behind the HRP conjugate. The HRP conjugate band eluted between fractions 11-16. The fractions containing the HRP conjugate are pooled in a 15 mL tissue culture with a screw cap. The HRP conjugate concentration

is determined at 403 nm after diluting an aliquot (usually 50  $\mu$ L + 650  $\mu$ L of buffer).

$$[\text{HRP conjugate}] \text{ (mg/mL)} = A_{403} \times 0.4 \times \text{D.F.}$$

5        The ultraviolet (UV) absorption spectrum is recorded between 320 and 220 nm. The presence of additional absorption peaks at 280 nm, 280 nm and 290 nm for caffeine-HRP, 1,7-DMX-HRP and 1,7-DMU-HRP conjugates, respectively, are indicators that the coupling proceeded  
10 as expected. After the above measurements, 5  $\mu$ L of a 4% thiomersal solution is added per mL of caffeine-HRP, 1,7-DMX-HRP or 1,7-DMU-HRP conjugate solution. The conjugates are stored at 4°C.

#### 15        Antibody Production

      Six mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) are used for antibody production. The protocol employed in this study was approved by the McGill University Animal Care  
20 Committee in accordance with the guidelines from the Canadian Council on Animal Care. An isotonic saline solution (0.6 mL) containing 240  $\mu$ g of KLH conjugated antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. Then, 0.5 mL of the emulsion (100  $\mu$ g of antigen) is  
25 injected per rabbit intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50  $\mu$ g of antigen emulsified in incomplete Freund's adjuvant. Blood is collected without anticoagulant in a vacutainer tube by venipuncture of the ear 10-14

days after boosting and kept at 4°C. After clotting, and centrifugation at 4°C, sodium azide is added to the antisera to a final concentration of 0.001% (1µL of a 1 % sodium azide solution per mL of antisera). Antisera are  
5 stored as 0.5 mL aliquots at -20 °C.

#### Antiserum titers

The wells of a microtiter plate are coated with 10 µg mL<sup>-1</sup> of bovine serum albumin-caffeine (or 1,7-dimethyl xanthine, 1,7-dimethyluric acid) conjugate in 100 mM sodium carbonate buffer, pH 9.6) overnight at 4° C (150 µL/well). They are then washed three times with TPBS (phosphate buffer saline containing 0.05 % Tween 20) using a Nunc Immuno Wash 12 autoclavable. Unoccupied sites are  
10 blocked by an incubation with 150 µL/well of TPBS containing 0.05 % porcine gelatin for 2 h at room temperature. The wells are washed three times with TPBS and 150 µL of antiserum diluted in TPBS are added. After 2 h at room temperature, the wells are washed three times  
15 with TPBS, and 100 µL of goat anti-rabbit IgGs-alkaline phosphatase conjugate diluted in PBS containing 1% BSA are added. After 1 h at room temperature, the wells are washed three times with TPBS and three times with water. To the wells are added 150 µL of a solution containing MgCl<sub>2</sub> (0.5  
20 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency is read at 405 nm with a microplate reader. The antibody titer is defined as the

25

dilution required to change the absorbance by one unit (1 au).

#### Isolation of IgG antibodies

5 Rabbit IgG antibodies against KLH conjugates are purified by affinity chromatography on a Protein A-Sepharose 4B column as follows. A 0.9 x 15 cm Pharmacia chromatographic column is packed with Protein A-Sepharose 4B suspension to a volume of 1 mL. The column is washed  
10 generously with a 0.01 M  $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$  buffer, pH 8.0 containing 0.15 M NaCl (PBS) and then washed with 3-4 mL of a 0.1 M trisodium citrate buffer, pH 3.0. The column is then washed generously with PBS. Then, 1 mL of rabbit antiserum is diluted with 1 mL PBS, and the resulting  
15 solution is slowly applied to the column. The column is washed with 15 mL PBS and eluted with a 0.1 M trisodium citrate buffer, pH 3.0. Three fractions of 2.2 mL are collected in 15-mL graduated tubes containing 0.8 mL of 1 M Tris-HCl buffer, pH 8.5. The purified rabbit IgG anti-  
20 bodies are stored at 4°C in the presence at 0.01 % sodium azide.

#### Competitive antigen ELISA

Buffers and water without additives are filtered  
25 through 0.45  $\mu\text{M}$  millipore filters and kept for one week, except the substrate buffer which is freshly prepared. BSA, antibodies, Tween<sup>TM</sup> 20 and horse radish peroxidase are added to buffers and water just prior to use. Urine  
30 samples are usually collected four hours after drinking a cup of coffee (instant or brewed with approximately 100 mg

of caffeine per cup) and stored at  $-20^{\circ}\text{C}$  as 1-mL aliquots in 1.5-mL microtubes. For the ELISA, the urine samples are diluted with isotonic sodium phosphate buffer, pH 7.5 (310 mosM) to give concentrations of caffeine, 1,7-DMX and 1,7-DMU no higher than  $3 \times 10^{-6}$  M in the microtiter plate wells. Wells of the ELISA plate are washed with a Nunc-Immuno wash 12 washer. Sixteen milliliters of a solution of  $6.6 \mu\text{g ml}^{-1}$  of isolated IgG antibodies is prepared in a 100 mM sodium carbonate buffer, pH 9.6, and 150  $\mu\text{L}$  of this solution is pipetted in each well of a microtiter plate using a eight channel pipet (Brinkmann Transferpette<sup>TM</sup>-8 50-200  $\mu\text{L}$ ) and 200 $\mu\text{L}$  Flex tips from Brinkmann). After coating the wells with antibodies at  $4^{\circ}\text{C}$  for 20 hours, the wells are washed 3 times with the isotonic sodium phosphate buffer containing 0.05% Tween<sup>TM</sup> 20 (IPBT) and properly drained by inverting the plate and absorbing the liquid on to a piece of paper towel. Thirty milliliters of a solution of a IPBT solution containing 1% BSA is prepared and 150  $\mu\text{L}$  of this solution is pipetted in each well using a eight channel pipet (Brinkmann Transferpette<sup>TM</sup>-8 50-200  $\mu\text{L}$ ) and 200  $\mu\text{L}$  yellow tips (Sarstedt yellow tips for P200 Gilson Pipetman). After 3 hours at room temperature, the wells are washed 3 times with IPBT solution and drained. Samples of 400  $\mu\text{L}$  for determination of caffeine, 1,7-DMX and 1,7-DMU are prepared in 1.5-mL microtubes using Sarstedt yellow tips and a P200 Gilson Pipetman. Then, 200  $\mu\text{L}$  of each sample are pipetted in duplicate in a Falcon 96-well microtest tissue culture plate according to the pattern shown in Figure 22, using

Sarstedt yellow tips and a P200 Gilson Pipetman. Using an eight channel pipet (Brinkmann Transferpette<sup>TM</sup>-8 50-200  $\mu$ L) and changing the tips of the eight channel pipet (200 $\mu$ L Flex tips from Brinkmann) at each row, 150  $\mu$ L of samples are transferred in the corresponding wells of a 96-well ELISA microtiter plate coated with antibodies. After the addition of the samples, the microtiter plates are covered and left standing at room temperature for 2 h. While the plate is left standing the substrate buffer without the hydrogen peroxide and o-phenylenediamine hydrochloride is prepared (25 mM citric acid and 50 mM sodium phosphate dibasic buffer, pH 5.0). The microtiter plate is washed 3 times with the IPBT solution and 3 times with a 0.05% Tween<sup>TM</sup> 20 solution and drained. Then, 50  $\mu$ L of hydrogen peroxide and 40 mg of o-phenylenediamine are added to the substrate buffer. One hundred fifty microliters (150  $\mu$ L) of the substrate buffer solution is then added to each well using a eight channel pipet (Brinkmann Transferpette<sup>TM</sup>-8 50-200  $\mu$ L) and 200 $\mu$ L Flex tips (Brinkmann). The microtiter plate is covered and shaken for 25-30 min at room temperature and the enzymatic reaction is stopped by adding 50  $\mu$ L/well of a 2.5 N HCl solution using an eight channel pipet (Brinkmann Transferpette<sup>TM</sup>-8 50-200  $\mu$ L) and 200 $\mu$ L Flex tips (Brinkmann). After gently shaking for 3 min., the absorbance is read at 490 nm with a microplate reader.

Standard solutions of Caffeine, 1,7-DMX and 1,7-Dimethyluric acid solutions for ELISA

5 A 100 mL stock solution of each of caffeine, 1,7-DMX and 1,7-DMU acid at concentrations of  $6.00 \times 10^{-4}$  M, in the 310 mosM sodium phosphate buffer, pH 7.5 (IPB) is prepared in a 100 mL volumetric flask. The solution is stirred to insure complete solubilization.

The stock solutions are stored as 1 mL aliquots at -20°C.

10 On the day of the ELISA, one aliquot is thawed and warmed up to room temperature.

The standard solutions of the above compounds are prepared as outlined in Table 15 below.



**TABLE 15****Standard Solutions**

Standard #	[Compound]	Composition
1	$6.00 \times 10^{-4}$ M	Stock solution
2	$2.00 \times 10^{-4}$ M	200 $\mu$ L S1 + 400 $\mu$ L IPB
3	$1.12 \times 10^{-4}$ M	200 $\mu$ L S1 + 868 $\mu$ L IPB
4	$6.00 \times 10^{-5}$ M	100 $\mu$ L S1 + 900 $\mu$ L IPB
5	$3.56 \times 10^{-5}$ M	60 $\mu$ L S1 + 951 $\mu$ L IPB
6	$2.00 \times 10^{-5}$ M	100 $\mu$ L S2 + 900 $\mu$ L IPB
7	$1.12 \times 10^{-5}$ M	100 $\mu$ L S3 + 900 $\mu$ L IPB
8	$6.00 \times 10^{-6}$ M	100 $\mu$ L S4 + 900 $\mu$ L IPB
9	$3.56 \times 10^{-6}$ M	100 $\mu$ L S5 + 900 $\mu$ L IPB
10	$2.00 \times 10^{-6}$ M	100 $\mu$ L S6 + 900 $\mu$ L IPB
11	$1.12 \times 10^{-6}$ M	100 $\mu$ L S7 + 900 $\mu$ L IPB
12	$6.00 \times 10^{-7}$ M	100 $\mu$ L S8 + 900 $\mu$ L IPB
13	$3.56 \times 10^{-7}$ M	100 $\mu$ L S9 + 900 $\mu$ L IPB
14	$2.00 \times 10^{-7}$ M	100 $\mu$ L S10 + 900 $\mu$ L IPB
15	$1.12 \times 10^{-7}$ M	100 $\mu$ L S11 + 900 $\mu$ L IPB
16	$6.00 \times 10^{-8}$ M	100 $\mu$ L S12 + 900 $\mu$ L IPB
17	$3.56 \times 10^{-8}$ M	100 $\mu$ L S13 + 900 $\mu$ L IPB
18	$2.00 \times 10^{-8}$ M	100 $\mu$ L S14 + 900 $\mu$ L IPB
19	$2.00 \times 10^{-9}$ M	100 $\mu$ L S18 + 900 $\mu$ L IPB
20	$2.00 \times 10^{-10}$ M	100 $\mu$ L S19 + 900 $\mu$ L IPB
21	$2.00 \times 10^{-11}$ M	100 $\mu$ L S20 + 900 $\mu$ L IPB
22	$2.00 \times 10^{-12}$ M	100 $\mu$ L S21 + 900 $\mu$ L IPB
23	$2.00 \times 10^{-13}$ M	100 $\mu$ L S22 + 900 $\mu$ L IPB

### Antibody Specificity

To ensure accuracy in the ELISA measurement of CYP1A2 phenotyping, the antibodies must have specificity for their individual caffeine metabolites, with little or no recognition of other derivatives. To ensure their selectivity an ELISA is performed with standard solutions of the compounds listed in Table 16. An ideal antibody specificity result is hypothesized with the Table 16 as well.

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**Table 16**

**Cross-reactivity of caffeine-Ab, 1,7-DMX-Ab and 1,7-DMU-Ab towards caffeine metabolites and structural analogs**

5

Compound	% Cross-reaction		
	Caffeine-Ab	1,7-DMX-Ab	1,7-DMU-Ab
Caffeine	100	0 <sup>a</sup>	0
<b>Xanthine</b>	<b>0</b>	<b>0</b>	<b>0</b>
Hypoxanthine	0	0	0
1-Methyl Xanthine	0	0	0
3-Methyl Xanthine	0	0	0
7-Methyl Xanthine	0	0	0
8-Methyl Xanthine	0	0	0
1,3-Dimethyl Xanthine <sup>b</sup>	0	0	0
1,7-Dimethyl Xanthine <sup>c</sup>	0	100	0
3,7-Dimethyl Xanthine <sup>d</sup>	0	0	0
Uric acid	0	0	0
1-Methyluric acid	0	0	0
3-Methyluric acid	0	0	0
7-Methyluric acid	0	0	0
1,3-Dimethyluric acid	0	0	0
1,7-Dimethyluric acid	0	0	100
3,7-Dimethyluric acid	0	0	0
1,3,7-Trimethyluric acid	0	0	0
Guanine	0	0	0
Uracil	0	0	0
AAU <sup>e</sup>	0	0	0
AAMU <sup>f</sup>	0	0	0
AADMU <sup>g</sup>	0	0	0

a. The number 0 indicates either an absence of inhibition or an inhibition no higher than 40% at the highest concentration tested in the ELISA ( $5 \times 10^{-3}$  M); concentrations of caffeine, 1,7-Dimethyl Xanthine and 1,7-Dimethyluric acid required for 50% inhibition in the competitive antigen ELISA will be determined; b, 1,3-Dimethyl Xanthine, theophylline; c, 1,7-Dimethyl Xanthine, paraxanthine; d, 3,7-Dimethyl Xanthine, theobromine; e, AAU, 5-acetamido-6-aminouracil; f, AAMU, 5-acetamido-6-amino-3-methyluracil; g, AADMU, 5-acetamido-6-amino-1,3-dimethylxanthine.

#### Results

Positive creation of antibodies against caffeine, 1,7-DMX, and 1,7-DMU can be seen by antibody titers of 30,000-100,000 as determined by the ELISA, strong precipitation lines after double immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin, and low cross-reactivity with other caffeine derivatives. These results constitute positive conditions for the development of a competitive antigen ELISA according to the methods described in the above section entitled Materials and Methods.

In accordance with one embodiment of the present invention, a competitive antigen ELISA is developed for CYP1A2 phenotyping using caffeine as the probe substrate. Contrary to current methods used for phenotyping, the assay is sensitive, rapid and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

## EXAMPLE II

Determination of Caffeine, 1,7-Dimethyl Xanthine (1,7-DMX) and 1,7-Dimethyluric acid (1,7-DMU) in urine samples with the ELISA kit

5

**Table 17****Content of the ELISA kit and conditions of storage**

Item	Unit	State	Amt.	Storage Conditions
Tween™ 20	1 vial	liquid	250 µL/vial	4°C
H <sub>2</sub> O <sub>2</sub>	1 vial	liquid	250 µL/vial	4°C
Caffeine-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMX-HRP	1 vial	liquid	250 µL/vial	4°C
1,7-DMU-HRP	1 vial	liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g /vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate (Caffeine-Ab)	2	Solid	-	4°C
Plate (1,7-DMX-Ab)	2	Solid	-	4°C
Plate (1,7-DMU-Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards (Caffeine)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMX)	14 vials	Liquid	200 µL	-20°C
Standards (1,7-DMU)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

Dilutions of urine samples for the determinations of

10 [Caffeine], [1,7-DMX] and [1X] by ELISA

The dilutions of urine samples required for determinations of caffeine, 1,7-DMX and 1,7-DMU are a function

of the sensitivity of the competitive antigen ELISA and of caffeine, 1,7-DMX and 1,7-DMU concentrations in urine samples. It is suggested to dilute the urine samples by a factor so that AAMU and 1X are about  $3 \times 10^{-6}$  M in the well of the microtiter plate.

**Table 18**  
**Microtube #**

Dilution Factor	20x	40x	50x	80x	100x	150x	200x	400x
Solution	1	2	3	4	5	6	7	8
Urine Sample (mL) <sup>a</sup>	500	250	200	125	100	66.7	50	25
10x diluted								
Buffer B (mL)	500	750	800	875	900	933.3	950	975

a: The microtubes containing the urine sample are vortexed before pipetting.

The diluted urine samples are stored at -20°C in a box for microtubes.

Buffer B: dissolve the content of 1 vial B/ 100 mL water

Determination of [caffeine], [1,7-DMX] and [1,7-DMU] in diluted urine samples by ELISA

#### Precautions

The substrate is carcinogenic. Wear surgical gloves when handling Buffer E (substrate buffer). Each sample is determined in duplicate. An excellent pipeting technique is required. When this technique is mastered the absorbency values of duplicates should be within less than 5%. Buffers C, D, E are freshly prepared. Buffer E-H<sub>2</sub>O<sub>2</sub> is prepared just prior to pipeting in the microtiter plate wells.

## Preparation of samples:

Prepare table 19 with a computer and print it. This table shows the contents of each well of a 96 well micro-titer plate. Enter the name of the urine sample (or number) at the corresponding well positions in Table 19. Select the dilution factor (D.F.) of each urine sample and enter at the corresponding position in Table 19. Enter the dilution of each urine sample with buffer B at the corresponding position in Table 19: for example a D.F. of 100 (100 $\mu$ L of 10x diluted urine sample + 900  $\mu$ L buffer B), enter 100/900. See "Dilutions of urine samples..." procedure described above for the preparation of the different dilutions. Prepare the different dilutions of the urine samples in 1.5 mL microtubes using a styrofoam support for 100 microtubes. Prepare Table 20 with a computer and print it. Using a styrofoam support (100 microtubes), prepare the following 48 microtubes in the order indicated in Table 20.

**Table 19****Positions of blanks, control and urine samples in a microtiter plate**

Sample	Well #	D.F.	Dil.	Sample	Well #	D.F.	Dil.
<b>Blank</b>	1-2	-		<b>Control</b>	49-50		-
<b>Control</b>	3-4	-		<b>8</b>	51-52		
<b>S1</b>	5-6	-		<b>9</b>	53-54		
<b>S2</b>	7-8	-		<b>10</b>	55-56		
<b>S3</b>	9-10	-		<b>11</b>	57-58		
<b>S4</b>	11-12	-		<b>12</b>	59-60		
<b>S5</b>	13-14	-		<b>13</b>	61-62		
<b>S6</b>	15-16	-		<b>14</b>	63-64		
<b>S7</b>	17-18	-		<b>15</b>	65-66		
<b>S8</b>	19-20	-		<b>16</b>	67-68		
<b>S9</b>	21-22	-		<b>17</b>	69-70		
<b>S10</b>	23-24	-		<b>Control</b>	71-72		-
<b>S11</b>	25-26	-		<b>18</b>	73-74		
<b>S12</b>	27-28	-		<b>19</b>	75-76		
<b>S13</b>	29-30	-		<b>20</b>	77-78		
<b>S14</b>	31-32	-		<b>21</b>	79-80		
<b>S15</b>	33-34	-		<b>22</b>	81-82		
<b>1</b>	35-36	-		<b>23</b>	83-84		
<b>2</b>	37-38	-		<b>24</b>	85-86		
<b>3</b>	39-40	-		<b>25</b>	87-88		
<b>4</b>	41-42	-		<b>26</b>	89-90		
<b>5</b>	43-44	-		<b>27</b>	91-92		
<b>6</b>	45-46	-		<b>28</b>	93-94		
<b>7</b>	47-48	-		<b>Blank</b>	95-96		-



**Table 20**  
**Content of the different microtubes**

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	Caffeine/1,7-DMX/1,7-DMU	27	9	Dil. Urine
4	S2	Caffeine/1,7-DMX/1,7-DMU	28	10	Dil. Urine
5	S3	Caffeine/1,7-DMX/1,7-DMU	29	11	Dil. Urine
6	S4	Caffeine/1,7-DMX/1,7-DMU	30	12	Dil. Urine
7	S5	Caffeine/1,7-DMX/1,7-DMU	31	13	Dil. Urine
8	S6	Caffeine/1,7-DMX/1,7-DMU	32	14	Dil. Urine
9	S7	Caffeine/1,7-DMX/1,7-DMU	33	15	Dil. Urine
10	S8	Caffeine/1,7-DMX/1,7-DMU	34	16	Dil. Urine
11	S9	Caffeine/1,7-DMX/1,7-DMU	35	17	Dil. Urine
12	S10	Caffeine/1,7-DMX/1,7-DMU	36	Control	Buffer B
13	S11	Caffeine/1,7-DMX/1,7-DMU	37	18	Dil. Urine
14	S12	Caffeine/1,7-DMX/1,7-DMU	38	19	Dil. Urine
15	S13	Caffeine/1,7-DMX/1,7-DMU	39	20	Dil. Urine
16	S14	Caffeine/1,7-DMX/1,7-DMU	40	21	Dil. Urine
17	S15	Caffeine/1,7-DMX/1,7-DMU	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

### Solutions

- 5 Buffer C: Dissolve the content of one vial C/50 mL water.  
Add 25 mL of Tween<sup>TM</sup> 20.

- Buffer D: Dissolve the content of one vial D/25 mL water.  
Add 25 mL of Tween™ 20.
- 5 0.05% Tween™ 20: Add 25 mL of Tween™ 20 in a 100 mL erlenmeyer flask containing 50 mL of water.
- 2.5N HCl: 41.75 mL of 12N HCl/200 mL water.  
Store in a 250 mL glass bottle.
- 10 Caffeine-HRP conjugate: Add 9 mL of Buffer C in a 15 mL glass test tube. Add 90 µL of caffeine-HRP stock solution.
- 1,7-DMX-HRP conjugate: Add 9 mL of Buffer C in a 15 mL glass test tube. Add 90 µL of 1,7-DMX-HRP stock solution.
- 15 1,7-DMU-HRP conjugate: Add 9 mL of the 2% BSA solution in a 15 mL glass test tube. Add 90 µL of 1,7-DMU-HRP stock solution.
- 20 Buffer E - H<sub>2</sub>O<sub>2</sub>: Dissolve the contents of 1 vial E-substrate/50 mL water. Add 25 µL of a 30% H<sub>2</sub>O<sub>2</sub> solution (prepared fresh).

**Table 21****Standard solutions of caffeine, 1,7-DMX and 1,7-DMU (diluted with buffer B)**

Standard	Caffeine	Standard	1,7-DMX	Standard	1,7-DMU
1	$1.12 \times 10^{-4}$ M	1	$1.12 \times 10^{-4}$ M	1	$1.12 \times 10^{-4}$ M
2	$6.00 \times 10^{-5}$ M	2	$6.00 \times 10^{-5}$ M	2	$6.00 \times 10^{-5}$ M
3	$3.56 \times 10^{-5}$ M	3	$3.56 \times 10^{-5}$ M	3	$3.56 \times 10^{-5}$ M
4	$2.00 \times 10^{-5}$ M	4	$2.00 \times 10^{-5}$ M	4	$2.00 \times 10^{-5}$ M
5	$6.00 \times 10^{-6}$ M	5	$6.00 \times 10^{-6}$ M	5	$6.00 \times 10^{-6}$ M
6	$3.56 \times 10^{-6}$ M	6	$3.56 \times 10^{-6}$ M	6	$3.56 \times 10^{-6}$ M
7	$2.00 \times 10^{-6}$ M	7	$2.00 \times 10^{-6}$ M	7	$2.00 \times 10^{-6}$ M
8	$1.12 \times 10^{-6}$ M	8	$1.12 \times 10^{-6}$ M	8	$1.12 \times 10^{-6}$ M
9	$6.00 \times 10^{-7}$ M	9	$6.00 \times 10^{-7}$ M	9	$6.00 \times 10^{-7}$ M
10	$3.56 \times 10^{-7}$ M	10	$3.56 \times 10^{-7}$ M	10	$3.56 \times 10^{-7}$ M
11	$2.00 \times 10^{-7}$ M	11	$2.00 \times 10^{-7}$ M	11	$2.00 \times 10^{-7}$ M
12	$1.12 \times 10^{-7}$ M	12	$1.12 \times 10^{-7}$ M	12	$1.12 \times 10^{-7}$ M
13	$6.00 \times 10^{-8}$ M	13	$6.00 \times 10^{-8}$ M	13	$6.00 \times 10^{-8}$ M
14	$3.56 \times 10^{-8}$ M	14	$3.56 \times 10^{-8}$ M	14	$3.56 \times 10^{-8}$ M
15	$2.00 \times 10^{-8}$ M	15	$2.00 \times 10^{-8}$ M	15	$2.00 \times 10^{-8}$ M

## 5 Conditions of the ELISA

Add 50  $\mu$ L/well of Caffeine-HRP (1,7-DMX-HRP or 1,7-DMU-HRP) conjugate solution starting from the last row. Add 50  $\mu$ L/well of diluted urine samples in duplicate, standards, blank with a micropipet (0-200  $\mu$ L), starting from well # 96 (see Table 22). The plate is covered and mixed gently by vortexing for several seconds. The plate is left at room temperature for 3 hours. The plate is then washed three times with 100  $\mu$ L/well buffer C, using a microtiter plate washer. The plate is then washed 3 times with 100  $\mu$ L/well 0.05% Tween<sup>TM</sup> 20 solution. Add 150  $\mu$ L/well of Buffer E - H<sub>2</sub>O<sub>2</sub> (prepared just prior to pipeting in the microtiter

plate wells). The plate is shaken for 20-30 min. at room temperature using an orbital shaker. Add 50  $\mu$ L/well of a 2.5N HCl solution. The plate is shaken 3 min. with the orbital shaker at room temperature. The absorbance of the wells is read with a microtiter plate reader at 490 nm. Print the sheet of data and properly label.

Calculation of the [caffeine], [1,7-DMX] and [1,7-DMU] in urine samples from the data

Draw Table 22 with a computer. Using the data sheet of the microtiter plate reader, enter the average absorbance values of blanks, controls (no free hapten present), standards and samples in Table 22. Draw the calibration curve on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma-plot (or other plot software). Find the [AAMU] (or [1X]) in the microtiter well of the unknowns from the calibration curve and enter the data in Table 23. Multiply the [caffeine] ([1,7-DMX] or [1,7-DMU] of the unknown by the dilution factor and enter the result in the corresponding cell of Table 23.

**Table 22****Average absorbance values of samples in the microtiter plate**

Sample	Well #	A <sub>490</sub>	Sample	Well #	A <sub>490</sub>
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

**Table 23**  
**Caffeine, 1,7-DMX and 1,7-DMU concentrations in urine samples**

Sample	D.F.	[Caffeine]	[caffeine] x D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			

**Table 24**  
**Composition of the different buffers**

Buffer	pH	Composition	Conc. (mM)	[P] (mM)
<b>A</b>	7.50	0.15629 g/100 mL $\text{NaH}_2\text{PO}_4$	11.325	71.424
		1.622 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	60.099	
		1.778 g/100 mL (total weight)		
<b>B</b>	7.50	0.1210191 g/100 mL $\text{NaH}_2\text{PO}_4$	8.769	49.999
		1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	41.23	
		1.2341 g/100 mL (total weight)		
<b>C</b>	7.50	1 g/ 100mL BSA		49.999
		0.1210191 g/100 mL $\text{NaH}_2\text{PO}_4$	8.769	
		1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	41.23	
		2.2341 g/100 mL (total weight)		
<b>D</b>	7.50	2 g/ 100mL BSA		49.999
		0.1210191 g/100 mL $\text{NaH}_2\text{PO}_4$	8.769	
		1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	41.23	
		3.2341 g/100 mL (total weight)		
<b>E</b>	5.00	0.52508 g/ 100mL of citric acid	25	-
		1.34848 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	50	
		40 mg/100 mL of o-phenylenedi- amine hydrochloride		
		1.913567 g/100 mL (total weight)		

5      A multi-determinant assay system is provided in accordance with the present invention. This assay system includes metabolite-specific binding agents for the

detection thereof in a biological sample. Such binding agents are preferably antibodies and the assay system is preferably an ELISA, as exemplified in the cases of NAT2 and CYP1A2 discussed herein above. Although the assay system of the present invention is exemplified in accordance with NAT2 and CYP1A2, it is not intended to be limited thereto. An assay system of the present invention, although not limited thereto, provides means to detect specific to determinants for at least the following drug metabolizing enzymes: CYP1A2, N-acetyltransferase-1 (NAT-1), N-acetyltransferase-2 (NAT-2), CYP2D6, CYP2A6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, UGT, GST, and SUT.

The present invention provides a convenient and effective tool for use in both a clinical and laboratory environment. The present invention is particularly suited for use by a physician in a clinic, whereby a fast and accurate result can be easily obtained. According to an embodiment of the present invention, a ready-to-use kit is provided for fast and accurate determination of a plurality of metabolic phenotypic determinants. The assay system and kit preferably employs antibodies specific to a plurality of metabolic pathways on a suitable substrate allowing for detection of the preferred metabolites in a biological sample of an individual after consumption of a corresponding probe substrate. In accordance with a preferred embodiment of the present invention, the assay system and kit of the present invention will provide means to determine metabolic determinants for at least the following enzymes, CYP1A2, N-acetyltransferase-1 (NAT-1),



N-acetyltransferase-2 (NAT-2), CYP2D6, CYP2A6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19.

### EXAMPLE III

#### 5 A Microarray ELISA for Identifying Multi-Determinant Metabolic Phenotypes

With the rapid growth of genomics in recent years, high-throughput technologies have emerged. DNA biochips, for example, are composed of densely packed probe arrays providing highly multiplexed and miniaturized molecular assays capable of rapidly genotyping an individual sample. These tools have been used for numerous molecular assays including high-throughput (HT) genotyping, differential gene expression, mutation detection and DNA sequencing. As a result, genetic material can be rapidly screened for numerous determinants of interest. For nearly 25 years, ELISA-based immunoassays have been the mainstay of diagnostic tests used for the detection of infectious disease. In more recent times, numerous researchers have refocused their attention on the continuing evolution of ELISA-based formats. In DNA-based arrays, high specificity is provided by Watson-Crick base pairing between complementary sequences under appropriate salt conditions using either oligonucleotides or cDNA-length probes. Alternatively, in a standard ELISA, specificity between antigen and antibody is governed by high affinity associations between an epitope on the antigen and its cognate binding site on the Fab portion of an IgG.

Antibodies can be implemented in a microarray ELISA assay for determining a metabolic phenotype. Antibodies of

the present invention may be polyclonal or monoclonal antibodies.

5 Numerous papers have been published recently in which protein microarrays - immunoassays are described. (Mendoza et al. Biotechniques 1999; Weiss et al. Clin Chem 2001; Silzel et al. Clin Chem 1998; US6083763; US6312960; US6331441). The accurate and reproducible array technology developed for spotting DNA samples has been adapted for use in spotting antibody solutions. The  
10 articles have demonstrated that multiple analytes can be assayed in one array if the antibodies selected have sufficient selectivity. The present invention can be adapted to all of the microarray formats.

According to a preferred embodiment of the present  
15 invention, the microarray immunoassay of the present invention will allow the rapid detection of multiple phenotypic determinants in an individual on one single assay plate. An example of the configuration of this plate is shown in Figs. 23 and 24. For example, as illustrated  
20 in Fig. 24, four 6X6 arrays can be provided in a single well. Accordingly, a multiple phenotyping assay for the 9 enzymes exemplified in accordance with the present invention can be performed in quadruplicate.

In brief, the system uses a microarray printer  
25 consisting of a 36-capillary array print head mounted to a high-resolution X-Y-Z positioning robot (Fig. 25). The print head consists of 36 capillaries precisely arranged in a 6x6 array. The print head can simultaneously deliver 36 different solutions. Each 6x6 array occupies a 3.24 mm<sup>2</sup>  
30 area with individual array elements occupying ~275 μm with

a center to center spacing of 300  $\mu\text{m}$ . This size allows for the placing of 4 6x6 arrays within a single well of a 96 well plate, producing 144 individual sites within the well and 13824 on a 96 well plate. The microarray printing process is very efficient, requiring a single milliliter of antigen solution to print more than 5 million arrays, based upon a standard spot volume of ~200 pl.

After printing the arrays are dried, the quality of the printing is rapidly confirmed by nondestructive imaging the dried protein-salt crystals using a high-resolution charge-coupled device (CCD) camera with ambient room-light illumination.

Once the quality is assured, the plates can be used to perform a microarray ELISA format (Mendoza et al., (1999) BioTechniques, 27(4):778-788.). For example, the plates are washed to remove any unbound antigen, non-specific binding blocked with casein, and then incubated with respective samples. The array detection is performed with alkaline phosphatase conjugates and the fluorescent alkaline phosphatase substrate ELF, as exemplified in Fig. 26. Imaging was performed using a scanning CCD Imager.

The main components of the CCD imager include: a peltier-cooled Pixel Vision camera consisting of a SpectraVideo front-illuminated CCD camera that has an attached Micro-Nikkor<sup>TM</sup> AF lens (60mm f/2.8D; Nikon, Melville, PA. USA). The CCD is mounted to a Z-axis drive that positions the CCD camera inline with a single microarray plate before image acquisition. The associated plate-carrier tray is mounted on an X-Y stage that

provides loading/unloading and scanning of slides containing the microarrays about the detection plane.

A method for performing a HT-ELISA of the present invention includes the administration of non-toxic probe substrates and identifying multiple determinants of an individual's spectrum of metabolic enzyme phenotypes, such as those listed in Table 4. In other words, non-toxic probe substrates are administered to activate metabolic pathways of interest and enzyme-specific determinants are identified to characterize an individual's phenotype. The individual to be phenotyped consumes these drugs, and the individual's urine collected 4 hours after consumption. The urine will be analyzed via the ELISA technology developed in the present invention. The urine samples may be monitored for the following probe substrate and/or metabolites (Figs. 1-9), and the molar (or chiral) ratios calculated to reveal the individual phenotypes.

#### Determination of Phenotypic Determinants in a Biological Sample

The advantage of the microarray ELISA over standard ELISA assays is the ability to accommodate multiple analytes per well, providing a greater yield of information per unit of sample input. The microarray ELISA system of the present invention will provide a practical and accurate means for determining a multi-determinant phenotype in both a clinical and laboratory environment. According to a preferred embodiment of the present invention, the microarray ELISA will provide means

for detecting determinant-specific metabolites for at least the following enzymes: CYP1A2, N-acetyltransferase-1 (NAT-1), N-acetyltransferase-2 (NAT-2), CYP2D6, CYP2A6, CYP2E1, CYP3A4, CYP2C9 and CYP2C19, UGT, GST, SUT. In  
5 this manner, the metabolic efficiency of an individual may be characterized prior to the prescription of a drug treatment regime, for example.

#### EXAMPLE IV

##### Phenotypic Determinants Identified by Rapid Immunoassays 10 (Dipstick) Technology

In rapid immunoassays the antibody or antigen reagents are bound to porous membranes, which react with positive samples while channeling excess fluids to a non-reactive part of the membrane. There are two common configurations,  
15 a lateral flow test where the sample is simply placed in a well and the results read immediately; and a flow through system, which requires placing the sample in a well, washing the well, and then finally adding an analyte-colloidal gold conjugate. The results can be read after a  
20 few minutes. One sample is tested per strip or cassette.

Rapid tests are faster than microtiter plate assays, require little sample processing, are often cheaper and generate yes/no answers without using any instrument, and hence they are often used in the field by non-laboratory  
25 people testing whole samples. However, standard rapid immunoassays are not as sensitive nor can they be used to accurately quantitate an analyte.

However, several novel technologies have been developed to allow quantitative measurements using a rapid

ELISA format. (US5942407; US4446232; US5695928; US5714389). The present invention is exemplified using the Rapid Analyte Measurement Platform (RAMP) technology, but is not limited thereto.

5 RAMP has been used to develop accurate quantitative tests for detecting thrombospodin, microalbumin, myoglobin and hepatitis B.

The RAMP system consists of two components: a disposable cartridge that houses an analyte-specific  
10 immunochromatographic strip, and a portable fluorescent reader that is used to quantify antibody-antigen complexes. The membrane strip component uses two populations of latex particles- each independently fluorescently labeled and tagged with monoclonal  
15 antibodies - to generate two independent signals during the assay reaction. One of these is the test reaction and the other is an independent internal standard.

The RAMP reader determines the absolute concentration of complexed latex particles. To perform this task, the  
20 reader calculates the ratio between the concentrations of latex particles in the test and internal standard reactions refers to an analyte specific calibration curve and converts the signal to a concentration. The RAMP technology has been developed to allow signal improvement  
25 resulting in a range of sensitivity comparable to ELISAs.

To perform a test, the operator places an unmetereed fluid sample containing a soluble analyte into the well of a test cartridge specific to the analyte of interest, and inserts the cartridge into the reader. Fig. 27 exemplifies

a test according to this embodiment of the present invention.

5 Contact Zone: Once in the cartridge the liquid sample is drawn by capillary action along the membrane through the contact zone. There it comes into contact with two populations of fluorescently labeled antibody-coated latex particles. Both populations of particles are coated with the same surface concentration of antibody, but are labeled with different dyes, and different antibodies. The  
10 test particles are labeled with an antibody against that analyte of interest. The internal standard particles are labeled with an "anti-rat" or "anti-mouse" antibody directed against a control reagent that does not react with the antibody directed against the analyte. When the  
15 liquid migrates through the contact zone, the colored latex particles are mobilized and carried along the membrane strip. If present in the sample, the target analyte binds covalently to the antibody on the test latex particles. The fluid sample, along with bound latex  
20 particles, unbound particles, and internal standard particles, is then transported by capillary action through the strip to the detection zones.

Detection Zone: Further along the membrane strip, the detection zone is embedded with a second immobilized  
25 antibody specific to the target analyte. If the fluid contains the target analyte, the antibody in the detection zone, which arrests the migration of the attached latex particles, will bind it. If no target analyte is present, the latex particles will migrate past the detection zone.  
30 The quantity of latex particles immobilized in the

detection zone is directly related to the concentration of the target analyte in the sample.

Internal Standard Zone: Past the detection zone is the internal standard zone, which is embedded with an immobilized mouse or rat immunoglobulin. The internal standard particles bind with this antigen and are arrested at this line.

### Results

The reader's two detectors separately measure the amount of fluorescence emitted by the complexes bound on the detection and internal standard zones. To establish a quantitative reading, the reader calculates the ratio between the two reactions. The internal standard corrects for membrane variability and provides an internal validation of the assay. Since the internal standard should always detect a consistent amount of fluorescence, any variation in this measurement should also be reflected in variation of the test measurement. For instance, membrane variations that cause a lower-than-normal reading on the internal standard should have the same effect on the test measurement. By calculating the final test result as a ratio between the two measurements, the RAMP system automatically accounts for such variations. No reading from the internal standard indicates that the test was not successfully completed, and acts as an automatic quality control.

Within 5 to 10 minutes, depending on which assay is being run, the assay is complete and the test result then appears on an LCD readout. The result can also be stored,



printed, or loaded to a laboratory or hospital information system.

This embodiment of the present invention is particularly suited to clinical use whereby a physician can quickly and accurately determine phenotypic determinants. In particular, in accordance with this embodiment, a dipstick or a qualitative detection instrument may be provided for detecting the presence of determinant specific metabolites in a biological sample.

10

#### EXAMPLE V

#### An Indirect Method of Identifying Multi-Determinant Metabolic Phenotypes by Genotyping

Indirect phenotyping by inference of phenotype from genotyping results is limited by the aforementioned restrictions. However, in some instances the use of indirect phenotyping can be beneficial. In some instances it may be sufficient to identify individuals with mutant alleles not producing the enzyme or producing a non-functional enzyme. This identification would allow physicians to avoid prescribing drugs harmful to those that can not metabolize them. As inducers or inhibitors of the enzyme can not affect the enzyme status of these individuals, they may be advised to avoid all of these drugs with no future phenotyping (indirect or direct) tests required.

25

Several methods have been developed for determining genotype.

#### Genotyping by PCR-RFLP:

This methodology can be briefly summarized as involving the PCR amplification of a region containing a gene mutation. The presence of the gene mutation is then  
5 detected by an altered pattern of digestion in the presence of a specific restriction enzyme. There are several variants of this methodology with multiple mutations being examined from one fragment, multiple restriction enzymes being used, or in the case where the  
10 mutation does not alter any naturally occurring restriction sites, artificial sites are introduced into the specific primers used in the PCR amplification (Taube, J., Halsall, D. and Baglin, T. (2000) Blood 96(5): 1816-1819).

15 This methodology is best used in cases where only a few mutations must be examined to obtain complete genotyping information.

#### Genotyping by PCR-ELISA:

20 This methodology relies on the detection of PCR reactions using a color producing reaction in a 96 well plate much as would be performed in a standard ELISA. Several formats of this basic protocol have been developed.

25 For example, the fragment of DNA containing the mutation of interest is amplified by PCR using digoxigenin labeled dUTP (DIG-11-dUTP) (Fig. 28). The PCR product is denatured and hybridized to a biotin-labeled oligonucleotide capture probe. The capture probe  
30 specifically recognizes the mutation sequence of interest. The biotin is then used to immobilize the PCR products

onto a well surface, of a streptavidin-coated microplate.  
The bound hybrids are with a standard ELISA enzyme  
(e.g.alkaline phosphatase)-conjugated with an anti-  
digoxigenin antibody and the enzyme substrate (e.g. para  
5 nitrophenylene phosphate, pNPP).

#### Advantages of PCR ELISA:

The main advantages of the PCR ELISA technique are:

10     *High sensitivity:* Detect PCR products with 10- to 100-  
fold greater sensitivity than fluorescent gel staining  
techniques.

*High specificity:* Selectively detects your PCR product  
with a sequence-specific biotin-labeled capture probe.

15     Discriminate single base pair mismatches or allelic  
variants (through optimization of the hybridization and  
washing conditions).

*Rapid, high throughput sample analysis:* Analyze up to  
96 samples in 1.0-4.5 hours. Simplify and streamline the  
20     procedure by using multichannel pipettors, automatic plate  
washers, and microplate readers.

#### Array-based Genotyping

The array-based system is very similar to the PCR-  
25     ELISA methodology however at a micro scale.

In brief, a multiplex PCR reaction is performed on a  
fragment with the mutations of interest. The PCR is  
performed with the digoxigen labeled dUTP nucleotide. The  
PCR samples are then added to the array, which consists of  
30     microspotted sequence-specific capture probe  
oligonucleotides. Only the PCR products with the sequence  
that is complementary to the capture probe will bind to

that specific spot. The binding of a PCR product is then detected with an anti-digoxin antibody conjugated to an enzyme (e.g. alkaline phosphatase) and incubation with the enzyme substrate (e.g. fluorescent ELF).

5        For example an array could be set up to genotype for CYP2C19 by examining the presence of wild type sequences as well as the two mutations, (CYP2C9\*2, C416T; CYP2C9\*3, A1061C), or for a more complex CYP enzyme with greater number of mutations.

#### 10        Genotype to Phenotype

15        Once a genotype has been assigned to an individual, the next step is the extrapolation of genotype to phenotype. This extrapolation uses the fact that studies have been performed to link the effect of mutations on enzymatic levels and/or activity. Therefore, an individual with two alleles with mutations linked to decreased activity could be assigned a poor metabolizing phenotype, 20 while those with two copies of alleles with mutations linked to an increased enzyme activity could be assigned an extensive metabolizing phenotype. These links are specific for each enzyme and each mutation of these enzymes.

25        If this method of phenotyping is to be used, then the individual's intake of enzyme inducers or inhibitors must be monitored carefully, as these compounds could alter the relationship between the genotype and the individual's actual phenotype.

30        It is fully contemplated that genotyping may be used in accordance with the present invention to identify

individual metabolic phenotypes and thus, find application in drug treatment screening.

#### EXAMPLE VI

#### 5 USE OF PHENOTYPIC DETERMINATION IN SELECTING DRUG TREATMENT REGIMES ON AN INDIVIDUAL BASIS

The knowledge of an individual's multiple phenotypic profile will allow physicians to:

- 10 a) Determine if the individual has a phenotype that allows the safe prescription of an individual drug and what is the optimal drug dose in terms of drug efficiency and drug safety.
- 15 b) Determine if the individual has a phenotype that allows the safe prescription of an individual class or genus of drugs and what is the optimal drug dose in terms of drug efficiency and drug safety.
- 20 c) Determine which drug of a plurality of drugs used for treating an individual's pathology or disease is the optimal drug in terms of drug efficiency and drug safety for that individual.

Some of the enzymes mentioned in the context of this invention have a clear bimodal distribution of metabolism, allowing the separation of the population into poor and  
25 extensive metabolizers. However, within each phenotypic group there is a wide variation in metabolic rates. It may be a naïve to regard all individuals with metabolic ratios greater than a predetermined cut off value as being equivalent. This attempt to classify the population in two  
30 or three phenotypic groups is even more difficult for

enzymes without a bimodal distribution. The classification of individuals into this limited classification may not allow for the complete exploitation of an individual's pattern of metabolism. In some cases this simple classification is sufficient, for example those individuals which are CYP2D6 deficient and at risk from severe complications if high doses of Prozac are prescribed. However, this simple classification would not allow for differential dosing of the extensive metabolizers as a function of the molar ratio calculated during determination of phenotype. If the simple classification of extensive CYP2D6 metabolizers was used, all individuals with a molar ratio of  $> 0.3$  (dextromethorphan as probe substrate) would receive the same dose. We are proposing the development of a dosing scale that would produce an increasing dose with increasing metabolic ratio, as exemplified in Fig. 29. If only the bimodal distribution is considered, only two possible doses can be prescribed.

#### Example A

##### Tramadol (ULTRAM)

Tramadol is a centrally acting analgesic drug that has an analgesic effect and a potency that ranges between weak opioids and morphine. Studies have suggested that while tramadol does have an opioid mechanism of action, it does not have a pronounced opioid side-effect profile.

Tramadol is metabolized into three main metabolites with CYP2D6 playing a primary role in the formation of the M1 metabolite.

Tramadol has a relative low affinity for the  $\mu$ -opioid receptor, (a factor of ~6000 weaker than morphine), however the M1 metabolite has an affinity for the  $\mu$ -opioid, which is about 200 times greater than the parent compound. Therefore, the opioid mechanism of action of tramadol appears to be mediated through the M1 metabolite.

As the formation of the M1 metabolite is dependent on the activity of CYP2D6, and the  $\mu$ -opioid mechanism of action is dependent on the M1 metabolite. It is highly possible that the  $\mu$ -opioid mechanism of action is dependent on the CYP2D6 enzyme activity. This premise was explored and validated in a study of 27 individuals (15 extensive and 13 poor metabolizers) (Poulsen et al. (1996) Clin Pharmacol & Ther 60(6):636-644). The individuals were dosed with tramadol (2mg/kg) or placebo and tested for pressure pain threshold, nociceptive reflex, cold pressor test and reaction time for auditory stimuli. In addition, individuals were interviewed about side effects including dizziness, tiredness and dry mouth. The study strongly suggested that tramadol was a better analgesic in extensive metabolizers as compared to poor metabolizers. Conversely, in extensive metabolizers tramadol caused side effects significantly more frequently than placebo whereas this was not the case for poor metabolizers.

Therefore, tramadol is an excellent example of a drug that would benefit from the determination of a metabolic phenotyping profile and the development of a dosing scale as per metabolic enzyme activity. If an individual was known to be a poor CYP2D6 metabolizer then a physician

would know that the effectiveness of tramadol would be greatly compromised and, if possible, prescribe an alternative therapy. In the case of extensive metabolizers, a dosing scheme may be developed that will  
5 allow for a sufficient dose for analgesic effect, yet avoid the significant increase in side effects. The higher an individual's enzymatic rate for CYP2D6, the lower the dose of tramadol is required to achieve an effective M1 concentration.

10 Example B

Tricyclic antidepressants (TCA)

The tricyclic antidepressants (TCA) class of agents includes amitriptyline, clomipramine, imipramine and  
15 nortriptyline. These compounds are used in the treatment of depression. Their use can be limited by the incidence of side effects which when minor can lead to low rates of compliance and when severe can be life threatening. TCA's can exert anticholinergic effects on the heart, which  
20 could cause a rise in heart rate and conduction delay. These effects are generally observed in individuals with underlying conduction problems.

To date tricyclic antidepressant dosing is performed by trial and error. The TCA is started at a low dose and  
25 increased until the therapeutic range, after that trial and error is often required. The most common cause of treatment failure for TCA is inadequate dosing.

The majority, if not all of the TCA class of agents, are metabolized significantly by the cytochrome P450  
30 enzyme systems. Of particular importance is CYP2D6 along with CYP1A2, CYP2C9 and CYP2C19. These enzymes play a



major role in the metabolism of the TCA agents. Many of the TCA's are metabolized by multiple phenotypes, and hence a multiple phenotyping procedure is required if the individual's phenotype is to be completely informative in regards to the metabolism of the TCA.

The knowledge of an individual's phenotype for the above mentioned CYPs may allow the physician to select among the TCAs, for the TCA agent, whose metabolic pathway is best matched to the metabolic profile of the individual. For example, clomipramine is metabolized by CYP1A2 and CYP2D6, while amitriptyline is metabolized by CYP2C9 and CYP2D6. If a individual was a poor CYP2C9, but an extensive CYP1A2 and CYP2D6 metabolizer, then the selection of clomipramine may be more suitable than amitriptyline. Accordingly, the need for a system and method for determining a multi-determinant phenotype on an individual basis is evident.

Once the TCA has been selected, knowledge of the enzyme activity of the particular enzymes responsible for the metabolism of selected TCA may help eliminate a degree of trial error in determining the correct dose of TCA. The metabolic profile of the individual individual will guide the physician to a more accurate range of dosing that is required. In addition, the increase in dosing accuracy may result in a decrease in side effects and hence lead to an increase in the compliance rate for the TCAs.

## Example C

## Organ Transplantation: Immunosuppression

The transplantation of solid organs has become the treatment of choice for end-stage renal, hepatic, cardiac and pulmonary disease. The field has progressed rapidly as a result of the development of safer and more effective immunosuppressive agents. The introduction in 1978 of cyclosporine, a specific and nonmyelotic immunosuppressant, changed heart and liver transplantation from research to service procedures and increased dramatically the success rate of renal transplantation. Since the introduction of cyclosporine other specific and nonmyelotic immunosuppressive agents have been discovered, notably Tacrolimus (formerly FK-506) and Sirolimus (also known as rapamycin). These three agents are a fundamental component of treatment required for a heart, liver or renal transplant.

While these agents are safer and more effective than the previous generation of immunosuppressors, their use is still associated with severe renal toxicity as a result of high serum levels. Low serum levels are related to decreased to efficacy, and consequently transplant rejection. These agents have a narrow therapeutic index. That is, there is a small range between blood concentrations that are too high and lead to side effects and those that are too low and lead to rejection.

The metabolism of all three agents is primarily through the CYP3A4 enzyme pathway. Therefore, an individual's level of CYP3A4 activity plays an important role in determining these agents' serum levels. In

addition, a study in 150 transplants suggested a link between decreased CYP3A4 activity and early cyclosporine-induced toxicity (Lemoine et al. (1993) Transplantation 56:1410-1414). Additionally, one individual in whom  
5 Tacrolimus-induced toxicity developed was found to have no detectable CYP3A4 activity before transplantation (Lemoine et al. (1994) Hepatology 20(6):1472-1477).

Therefore, the monitoring of the individual's (and possibly the donor's) level of CYP3A4 activity prior to  
10 transplantation may allow a more accurate selection of a dose of the specific and nonmyelotoxic agent being used. For example, if the individual has extremely low levels of CYP3A4, then the agent will not be metabolized and the blood levels will be high, and so a lower starting dose  
15 may be selected. Conversely, a individual with high levels of CYP3A4 activity may be given a higher dose, as the agent will be rapidly removed from their system.

The knowledge of an individual's multiple phenotypes will allow the detection of drug(s) that could cause  
20 significant side effects or be inefficient in individuals with a specific phenotypic profile. In addition, the phenotypic profile will allow the development of an individualized dosing scheme with dose related to level of enzyme activities. The implementation of the multi-  
25 determinant phenotyping profile in treatment and dosing selection will lead to a marked decrease in side effects and increase in therapeutic efficiency.

## EXAMPLE VII

USE OF PHENOTYPIC DETERMINATION FOR SCREENING CANDIDATES  
FOR PARTICIPATING IN CLINICAL TRIALS

5 New drug entities go through rigorous clinical trials  
prior to their approval for use in humans. These clinical  
trials are extremely lengthy and costly. During the course  
of clinical testing, many promising new drug candidates  
are abandoned due to unacceptable toxicity profiles. In  
10 some cases the unacceptable toxicity occurs only in a  
minority of the general individual population, those who  
possess a specific metabolic phenotype. Unfortunately,  
the ability to select defined individual populations for  
clinical trials has not been available on a routine basis,  
and this has resulted in the early termination of trials  
15 on otherwise promising new drug candidates.

In order to gain approval from a governing regulatory  
body (e.g. FDA) a drug must be proven to be safe and  
effective. This currently involves the testing of the drug  
in healthy normal volunteers and in individuals with the  
20 disease the drug is designed to treat. Huge numbers of  
individuals are involved and these trials can take upwards  
of 7 years to complete. The reason for the large number of  
individuals is to reach statistical significance to prove  
the safety and efficiency of the drug.

25 The highly variable inter-individual rates of  
metabolism for some drugs, in which the variability is  
related to side-effects and/or efficacy, the inclusion of  
individuals with a phenotype making them prone to decrease  
drug response or prone to increased adverse effects can  
30 result in an overall decrease in the trials drug

efficiency ratings or safety profiles. If this decrease in response rate or decreased safety profile is significant the drug may not be able to gain regulatory approval.

Therefore, screening by phenotyping prior to admission in to a clinical trial allows a decreased number of individuals required and may potentially allow the approval of drugs that otherwise would lack satisfactory response rates or safety profiles.

10 Example: Amonafide

The integration of phenotyping tests into the drug development process will allow for a decreased number of individuals participating in a drug treatment testing trial. This decrease in individual number will result in decreased costs and will allow the drug to reach the market faster.

An example of the power of metabolic phenotyping for individualizing therapy is evident in the case of the anti-cancer agent Amonafide. Amonafide is metabolized by NAT2. A phase II trial was conducted using a standard (300mg/m<sup>2</sup>) dose for all individuals. During this trial, two women (both oriental) died from severe myelosuppression; both of these women were extremely fast acetylators. The overall response rate for the trial was 18%, however it was noted that in those individuals considered to be properly dosed, a response rate of 30-50% was observed. It was noted that 300mg/m<sup>2</sup> - the dose used in the phase II trials, was an inappropriate dose for essentially all individuals, as fast acetylators would be expected to experience the most severe toxicity and slow acetylators

may be significantly underdosed. However, as a result of two deaths and the unsatisfactory response rate, to the best of our knowledge the development of Amonafide has been abandoned. The integration of pre-trial phenotyping could have avoided the loss of life and increased the response rate to a satisfactory level.

Pre-trial screening would involve the phenotyping of all individuals prior to inclusion in the trial. The phenotype status could then be used to identify those individuals at high risk for SAE's (severe adverse effects) and ensure that they were not included in the trial. The remaining individuals would then be treated with drug doses customized in correlation to their level of NAT2 activity. The customized doses would ensure that the individuals were receiving a safe efficacious treatment.

#### EXAMPLE VIII

##### USE OF PHENOTYPIC DETERMINATION IN RATIONAL DRUG DESIGN

The use of the phenotypic profiles in drug development may lead to more effective treatments with better safety profiles.

The use of phenotypic determination in studies to correlate toxicity and efficacy can lead to understanding of the effect of metabolism on incidence of toxicity or lack of efficacy.

If a compound is known to be converted to its metabolite by a specific enzyme, and a phenotypic study can directly correlate the incidence of toxicity to the level of the metabolic enzyme's activity, and hence the level of metabolite formed, the developers of this drug

may want to utilize an alternative approach. For example, the active metabolite might be developed as a drug on its. Even though the active metabolite is correlated to toxicity, if the active compound is delivered directly and is not affected by intra-individual variations in metabolism then controlling the concentration of the active compound may be more manageable and hence control the toxicity.

An alternate approach to rational drug design is in the combination of drugs with inhibitors or inducers. For example, if the therapeutic range of a drug is quite high and the drug is metabolized by CYP3A4, then if a person is an extensive CYP3A4 metabolizer, high doses of the drug may be necessary to reach the therapeutic range. If the use of high doses is not feasible (for reasons such as a difficulty in drug delivery, high costs etc...) then a CYP3A4 inhibitor may be used in conjunction with the therapeutic drug. This combination therapy will allow the generation of higher therapeutic drug levels without the necessity of higher drug doses. The knowledge of an individual's phenotype prior to the drug dosing is essential for this form of combination therapy.

A third example of the use of phenotyping in rational drug design can be exemplified by the use of cyclophosphamide in cancer individuals. Recent studies using direct phenotyping have demonstrated that the progression of cancer in many individuals results in the generation of the poor CYP2C19 metabolic status in individuals who genotypically are extensive metabolizers. As CYP2C19 is involved in the metabolism of

cyclophosphamide, the observed reduction in CYP2C19 activity in cancer individuals is a clinically relevant finding (Williams et al. (2000) Br J Clin Pharmacol 49: 485-488). A similar effect was observed for the

5 acetylation status of AIDS individuals. It was observed that disease progression in HIV infection and AIDS caused a significant number of individuals to become slow acetylators when they were genotypically fast (O'Neil et al. (1997) Clinical Pharmacol & Therap 62(3):261-270).

10 Therefore, phenotypic studies of specific individuals can lead to the identification of specific metabolic phenotypes that are associated with disease progression. The development of drugs to treat these diseases can be chosen to reflect these alterations. For example, if an

15 HIV therapy requires acetylation for activation, then this drug may not be as effective as believed. The HIV/AIDS individual population contains a higher percentage of slow acetylators, in whom the level of drug activation may not be sufficient to reach the therapeutic range required for

20 effective treatment.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or

25 adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features



hereinbefore set forth, and as follows in the scope of the appended claims.

2025 RELEASE UNDER E.O. 14176